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FILE 'JICST-EPLUS' ENTERED AT 12:36:23 ON 28 FEB 2000
FILE 'WPIDS' ACCESS NOT AUTHORIZED
FILE 'JAPIO' ENTERED AT 12:36:23 ON 28 FEB 2000

=> s (mva or ankara) and recombinant and virus

L1          179 (MVA OR ANKARA) AND RECOMBINANT AND VIRUS

=> dup rem l1

PROCESSING COMPLETED FOR L1
L2           63 DUP REM L1 (116 DUPLICATES REMOVED)

=> d bib ab 1-63


L2   ANSWER 1 OF 63  CAPLUS  COPYRIGHT 2000 ACS        DUPLICATE 1
AN   2000:99172  CAPLUS
TI   Use of apathogenic vaccinia virus MVA expressing EHV-1
     gC as basis of a combined recombinant MVA/DNA
     vaccination scheme
AU   Huemer, Hartwig P.; Strobl, Birgit; Nowotny, Norbert
CS   Institute of Hygiene, University Innsbruck, Innsbruck, A-6010, Austria
SO   Vaccine (2000), 18(14), 1320-1326
     CODEN: VACCDE; ISSN: 0264-410X
PB   Elsevier Science Ltd.
DT   Journal
LA   English
AB   The nonreplicating chicken adapted vaccinia virus strain
     MVA was used in a combined vaccine scheme.  Using the equine
     herpesvirus type 1 (EHV-1) encoded complement-receptor glycoprotein C as
     antigen, only poor antibody response was induced by exclusive vaccination
     with DNA plasmids.  The administration of recombinant
     MVA followed by plasmid immunization elicited both humoral and
     cellular immune responses in hamster comparable to EHV-1 full
     virus vaccines.  Our results indicate that recombinant
     constructs based on MVA represent a safe and efficient way to
     overcome problems of poor immunogenicity of certain antigens upon i.m. DNA
     vaccination, thus replacing sophisticated adjuvants or application
     methods, which are not readily applicable in routine practice.

L2   ANSWER 2 OF 63  MEDLINE                          DUPLICATE 2
AN   2000091348     MEDLINE
DN   20091348
TI   Biology of attenuated modified vaccinia virus Ankara
     recombinant vector in mice: virus fate and activation of
     B- and T-cell immune responses in comparison with the Western Reserve
     strain and advantages as a vaccine.
AU   Ramirez J C; Gherardi M M; Esteban M
CS   Department of Molecular and Cellular Biology, Centro Nacional de
     Biotecnologia, CSIC, Campus Universidad Autonoma, 28049 Madrid, Spain.
SO   JOURNAL OF VIROLOGY, (2000 Jan) 74 (2) 923-33.
```

Journal code: KCV. ISSN: 0022-538X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 200004
EW 20000402
AB The modified vaccinia **virus Ankara** (**MVA**) strain is a candidate vector for vaccination against pathogens and tumors, due to safety concerns and the proven ability of recombinants based on this vector to trigger protection against pathogens in animals. In this study we addressed the fate of the **MVA** vector in BALB/c mice after intraperitoneal inoculation in comparison with that of the replication-competent Western Reserve (WR) strain by measuring levels of expression of the reporter luciferase gene, the capability to infect target tissues from the site of inoculation, and the length of time of **virus** persistence. We evaluated the extent of humoral and cellular immune responses induced against the **virus** antigens and a **recombinant** product (beta-galactosidase). We found that **MVA** infects the same target tissues as the WR strain; surprisingly, within 6 h postinoculation the levels of expression of antigens were higher in tissues from **MVA**-infected mice than in tissues from mice infected with wild-type **virus** but at later times postinoculation were 2 to 4 log units higher in tissues from WR-infected mice. In spite of this, antibodies and cellular immune responses to viral vector antigens were considerably lower in **MVA** -inoculated mice than in WR **virus**-inoculated mice. In contrast, the cellular immune response to a foreign antigen expressed from **MVA** was similar to and even higher than that triggered by the **recombinant** WR **virus**. **MVA** elicited a Th1 type of immune response, and the main proinflammatory cytokines induced were interleukin-6 and tumor necrosis factor alpha. Our findings have defined the biological characteristics of **MVA** infection in tissues and the immune parameters activated in the course of **virus** infection. These results are of significance with respect to optimal use of **MVA** as a vaccine.

L2 ANSWER 3 OF 63 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 3
AN 2000:84621 CAPLUS
TI Induction of **recombinant** gene expression in stably transfected cell lines using attenuated vaccinia **virus MVA** expressing T7 RNA polymerase with a nuclear localization signal
AU Huemer, H. P.; Strobl, B.; Shida, H.; Czerny, C. P.
CS Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria
SO J. Virol. Methods (2000), 85(1-2), 1-10
CODEN: JVMEDH; ISSN: 0166-0934
PB Elsevier Science B.V.
DT Journal
LA English
AB There are major drawbacks using vaccinia **virus** (VV) expressing T7 polymerase for eukaryotic expression. VV is infectious for humans and due to cytosolic replication of Poxviridae, transient transfection of T7 promoter contg. plasmids is necessary, which varies in efficiency. Several improvements have been introduced to this system to enhance expression of herpes viral glycoproteins. Stably transfected cell lines were generated with an EBV-based episomal plasmid vector which can be pushed to increasing copy nos. under selective pressure. The avirulent vaccine **MVA** strain was adopted to generate a safe lab. vector for inserting the bacteriophage T7 RNA polymerase gene with (+) or without (-)a nuclear localisation signal. Constructs were designed for recombination into the VV haemagglutinin gene as recombinants could not be isolated successfully when inserting into the **MVA** thymidine kinase locus. Both T7 **MVA** recombinants induced foreign protein expression in transiently transfected cells but only the T7-/+ **MVA** induced target protein expression in stably transfected cells. The level

of protein expression by this induction mechanism was comparable to, or superior to levels obtained withVV recombinants expressing the gene under control of the VV 11 k IE promoter. The results suggests that the T7+ **MVA virus** can be used to induce gene expression in stable **recombinant** cell lines and offers an attractive and safe alternative to other inducible eucaryotic expression systems.

L2 ANSWER 4 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1999:224745 CAPLUS
DN 130:251219
TI Dengue **virus** antigens and treatment of dengue fever
IN Drexler, Ingo; Sutter, Gerd; Cardosa, Mary Jane; Hooi, Tio Phaik
PA Bavarian Nordic Research Institute A/S, Den.; GSF-Forschungszentrum Fur Umwelt Und Gesundheit G.m.b.H.; Universiti Malaysia Sarawak; Venture Technologies SDN BHD
SO PCT Int. Appl., 34 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 9915692	A2	19990401	WO 1998-EP6009	19980921
WO 9915692	A3	19990624		
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
AU 9910249	A1	19990412	AU 1999-10249	19980921
PRAI MY 1997-9704411	19970923			
WO 1998-EP6009	19980921			

AB The authors disclose monoclonal antibodies recognizing antigenic epitopes capable of eliciting neutralizing antibodies which protect against infection by dengue viruses. The elicited neutralizing antibodies are not capable of enhancement of dengue **virus** infection. The protective antigenic epitopes were selected by phage display. In addn., the authors describe the construction of **recombinant** vaccinia **virus** vaccine (i.e., modified vaccinia **virus** **Ankara** [**MVA**]) encoding for and capable of expressing dengue **virus** antigen.

L2 ANSWER 5 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1999:127038 CAPLUS
DN 130:178353
TI Vaccine comprising a live **recombinant** non-replicating **virus**
IN Small, Parker A., Jr.; Bender, Bradley Stephen; Meitin, Catherine Ann; Moss, Bernard
PA University of Florida, USA; United States Dept. of Health and Human Services
SO PCT Int. Appl., 57 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 9907869	A1	19990218	WO 1997-US13836	19970805
W: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR,				

GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG

AU 9739100 A1 19990301 AU 1997-39100 19970805

PRAI WO 1997-US13836 19970805

AB The invention pertains to a novel **recombinant** vaccinia **virus** (or canary pox **virus**) vaccine for use in immunizing animals and humans against disease. The vaccine comprises a live vaccinia/canary pox **virus** or a replication deficient mutant **virus** capable of expressing a single or multiple heterologous genes or gene fragments. In a preferred embodiment, the **recombinant virus** is contained in an orally-administered package that will only dissolve in the host animal's gut. The invention also pertains to a method of inducing a broad protective immune response through the oral, intranasal, or other mucosal means of administration of the **recombinant virus** vaccine.

L2 ANSWER 6 OF 63 MEDLINE DUPLICATE 4

AN 1999329216 MEDLINE

DN 99329216

TI The H gene of rodent brain-adapted measles **virus** confers neurovirulence to the Edmonston vaccine strain.

AU Duprex W P; Duffy I; McQuaid S; Hamill L; Cosby S L; Billeter M A; Schneider-Schaulies J; ter Meulen V; Rima B K

CS School of Biology and Biochemistry, The Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland, United Kingdom.. p.duprex@qub.ac.uk

SO JOURNAL OF VIROLOGY, (1999 Aug) 73 (8) 6916-22.
Journal code: KCV. ISSN: 0022-538X.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals; Cancer Journals

EM 199910

EW 19991004

AB Molecular determinants of neuropathogenesis have been shown to be present in the hemagglutinin (H) protein of measles **virus** (MV). An H gene insertion vector has been generated from the Edmonston B vaccine full-length infectious clone of MV. Using this vector, it is possible to insert complete H open reading frames into the parental (Edtag) background. The H gene from a rodent brain-adapted MV strain (CAM/RB) was inserted into this vector, and a **recombinant virus** (EdtagCAMH) was rescued by using a modified vaccinia **virus** which expresses T7 RNA polymerase (**MVA**-T7). The **recombinant virus** grew at an equivalent rate and to similar titers as the CAM/RB and Edtag parental viruses. Neurovirulence was assayed in a mouse model for MV encephalitis. Viruses were injected intracerebrally into the right cortex of C57/BL/6 suckling mice. After infection mice inoculated with the CAM/RB strain developed hind limb paralysis and ataxia. Clinical symptoms were never observed with an equivalent dose of Edtag **virus** or in sham infections. Immunohistochemistry (IHC) was used to detect viral antigen in formalin-fixed brain sections. Measles antigen was observed in neurons and neuronal processes of the hippocampus, frontal, temporal, and olfactory cortices and neostriatum on both sides of symmetrical structures. Viral antigen was not detected in mice infected with Edtag **virus**. Mice infected with the **recombinant virus**, EdtagCAMH, became clinically ill, and **virus** was detected by IHC in regions of the brain similar to those in which it was detected in animals infected with CAM/RB. The EdtagCAMH infection had, however, progressed much less than the CAM/RB **virus** at 4 days postinfection. It therefore appears that additional determinants are encoded in other regions of the MV genome which are required for full neurovirulence equivalent to CAM/RB. Nevertheless, replacement of the H gene alone is sufficient to cause neuropathology.

L2 ANSWER 7 OF 63 MEDLINE DUPLICATE 5

AN 1999446898 MEDLINE

DN 99446898

TI Modified vaccinia **virus Ankara** for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen A*0201-restricted cytotoxic T cells in vitro and in vivo.
AU Drexler I; Antunes E; Schmitz M; Wolfel T; Huber C; Erfle V; Rieber P; Theobald M; Sutter G
CS GSF-Institute for Molecular Virology, Munich, Germany.. drexler@gsf.de
SO CANCER RESEARCH, (1999 Oct 1) 59 (19) 4955-63.
Journal code: CNF. ISSN: 0008-5472.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 200001
EW 20000104
AB Vaccination with tumor-associated antigens is a promising approach for cancer immunotherapy. Because the majority of these antigens are normal self antigens, they may require suitable delivery systems to promote their immunogenicity. A **recombinant** vector based on the modified vaccinia **virus Ankara** (**MVA**) was used for expression of human tyrosinase, a melanoma-specific differentiation antigen, and evaluated for its efficacy as an antitumor vaccine. Stable **recombinant** viruses (**MVA**-hTyr) were constructed that have deleted the selection marker lacZ and efficiently expressed human tyrosinase in primary human cells and cell lines. Tyrosinase-specific human CTLs were activated in vitro by **MVA**-hTyr-infected, HLA-A*0201-positive human dendritic cells. Importantly, an efficient tyrosinase- and melanoma-specific CTL response was induced in vitro using **MVA**-hTyr-infected autologous dendritic cells as activators for peripheral blood mononuclear cells derived from HLA-A*0201-positive melanoma patients despite prior vaccination against smallpox. Immunization of HLA-A*0201/Kb transgenic mice with **MVA**-hTyr induced A*0201-restricted CTLs specific for the human tyrosinase-derived peptide epitope 369-377. These in vivo primed CTLs were of sufficiently high avidity to recognize and lyse human melanoma cells, which present the endogenously processed tyrosinase peptide in the context of A*0201. Tyrosinase-specific CTL responses were significantly augmented by repeated vaccination with **MVA**-hTyr. These findings demonstrate that HLA-restricted CTLs specific for human tumor-associated antigens can be efficiently generated by immunization with **recombinant** **MVA** vaccines. The results are an essential basis for **MVA** -based vaccination trials in cancer patients.

L2 ANSWER 8 OF 63 SCISEARCH COPYRIGHT 2000 ISI (R)
AN 1999:312701 SCISEARCH
GA The Genuine Article (R) Number: 187BP
TI Mucosal vaccination overcomes the barrier to **recombinant** vaccinia immunization caused by preexisting poxvirus immunity
AU Belyakov I M; Moss B; Strober W; Berzofsky J A (Reprint)
CS NCI, MOL IMMUNOGENET & VACCINE RES SECT, METAB BRANCH, NIH, BLDG 10, ROOM 6B-12, BETHESDA, MD 20892 (Reprint); NCI, MOL IMMUNOGENET & VACCINE RES SECT, METAB BRANCH, NIH, BETHESDA, MD 20892; NIAID, VIRAL DIS LAB, NIH, BETHESDA, MD 20892; NIAID, MUCOSAL IMMUN SECT, CLIN INVEST LAB, NIH, BETHESDA, MD 20892
CYA USA
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (13 APR 1999) Vol. 96, No. 8, pp. 4512-4517.
Publisher: NATL ACAD SCIENCES, 2101 CONSTITUTION AVE NW, WASHINGTON, DC 20418.
ISSN: 0027-8424.
DT Article; Journal
FS LIFE
LA English
REC Reference Count: 28
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB Overcoming preexisting immunity to vaccinia **virus** in the

adult population is a key requirement for development of otherwise potent **recombinant** vaccinia vaccines. Based on our observation that s.c. immunization with vaccinia induces cellular and antibody immunity to vaccinia only in systemic lymphoid tissue and not in mucosal sites, we hypothesized that the mucosal immune system remains naive to vaccinia and therefore amenable to immunization with **recombinant** vaccinia vectors despite earlier vaccinia exposure, We show that mucosal immunization of vaccinia-immune BALB/c mice with **recombinant** vaccinia expressing HIV gp160 induced specific serum antibody and strong HIV-specific cytotoxic T lymphocyte responses. These responses occurred not only in mucosal but also in systemic lymphoid tissue, whereas systemic immunization was ineffective under these circumstances. In this context, intrarectal immunization was more effective than intranasal immunization. Boosting with a second dose of **recombinant** vaccinia was also more effective via the mucosal route. The systemic HIV-specific cytotoxic T lymphocyte response was enhanced by coadministration of IL-12 at the mucosal site. These results also demonstrate the independent compartmentalization of the mucosal versus systemic immune systems and the asymmetric trafficking of lymphocytes between them. This approach to circumvent previous vaccinia immunity may be useful for induction of protective immunity against infectious diseases and cancer in the sizable populations with preexisting immunity to vaccinia from smallpox vaccination.

L2 ANSWER 9 OF 63 SCISEARCH COPYRIGHT 2000 ISI (R)
AN 1999:755030 SCISEARCH
GA The Genuine Article (R) Number: 241BZ
TI A model for vaccinia **virus** pathogenesis and immunity based on intradermal injection of mouse ear pinnae
AU Tscharke D C; Smith G L (Reprint)
CS UNIV OXFORD, SIR WILLIAM DUNN SCH PATHOL, S PARKS RD, OXFORD OX1 3RE, ENGLAND (Reprint); UNIV OXFORD, SIR WILLIAM DUNN SCH PATHOL, OXFORD OX1 3RE, ENGLAND
CYA ENGLAND
SO JOURNAL OF GENERAL VIROLOGY, (OCT 1999) Vol. 80, Part 10, pp. 2751-2755. Publisher: SOC GENERAL MICROBIOLOGY, MARLBOROUGH HOUSE, BASINGSTOKE RD, SPENCERS WOODS, READING RG7 1AE, BERKS, ENGLAND. ISSN: 0022-1317.
DT Article; Journal
FS LIFE
LA English
REC Reference Count: 17
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB Vaccinia **virus** (VV) proteins that interfere with the host response to infection are of interest because they provide insight into **virus**-host relationships and may affect the safety and immunogenicity of **recombinant** VV (rVV) vaccines. Such vaccines need assessment in animal models and with this aim a model of VV infection based on intradermal injection of BALB/c ear pinnae was developed and characterized. In this model, the outcome of infection is affected by the dose of **virus** inoculated but **virus** spread is minimal and the mice suffer no signs of systemic illness. Cellular and humoral immune responses to these infections were measured readily and were independent of **virus** dose over a 100-fold range. Thus the model seems suitable for the analysis of the safety and immunogenicity of VV mutants lacking specific immunomodulatory proteins or bearing foreign antigens.

L2 ANSWER 10 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1999:614625 CAPLUS
DN 132:769
TI Endothelial cell DNA transfer and expression using petri dish electroporation and the nonreplicating vaccinia **virus**/T7 RNA polymerase hybrid system
AU Lewis, E. W.; Rudo, T. J.; John, M. A. Rahman St.; Chu, J. L.; Heinze, A. W.; Howard, B. H.; Engleka, K. A.
CS Department of Physiology, Jefferson Medical College, Thomas Jefferson

University, Philadelphia, PA, 19107-6799, USA
SO Gene Ther. (1999), 6(9), 1617-1625
CODEN: GETHEC; ISSN: 0969-7128
PB Stockton Press
DT Journal
LA English
AB The nonreplicating vaccinia **virus MVA**/T7 RNA polymerase hybrid system was tested with Petri dish electroporation for ectopic gene expression in human umbilical vein endothelial cells (HUVECs). A range of voltages (150-450 V), pulse times (10-40 ms), DNA concns. (0-20 .mu.g/mL) and infection levels (0-15 multiplicities of infection) were tested for effects on T7 promoter-directed chloramphenicol acetyltransferase (CAT) activity after **MVA**/T7RP infection. **MVA**/T7RP-directed expression was transient and at least 10,000-fold in excess of nonviral, cytomegalovirus enhancer-directed expression. Use of a Petri dish electrode with the **MVA**/T7RP system showed increased viability compared with a cuvette electrode. Overexpression of interleukin-2 alpha subunit (IL2R.alpha.) protein followed by anti-IL2R.alpha.-directed magnetic immunoaffinity cell sorting allowed isolation of the transfected population. The high fidelity of cellular sorting was shown by segregation of CAT activity in the IL2R.alpha.-sorted population after transfection of T7 promoter-directed bicistronic IL2R.alpha./CAT DNA. Expression of a panel of proteins including the fluorophore green fluorescent protein as detected by fluorescence microscopy and p21cip1, p27kip1, pp60c-src, FGF-1, pRb, p107 and pRb2/p130 proteins was also achieved. Thus, use of the nonreplicating vaccinia **virus**/T7 RNA polymerase expression system with Petri dish electroporation is feasible for certain applications for the manipulation of HUVECs by gene transfer.

L2 ANSWER 11 OF 63 MEDLINE DUPLICATE 6
AN 1999246333 MEDLINE
DN 99246333
TI Comparison of the immunogenicity and efficacy of a replication-defective vaccinia **virus** expressing antigens of human parainfluenza **virus** type 3 (HPIV3) with those of a live attenuated HPIV3 vaccine candidate in rhesus monkeys passively immunized with PIV3 antibodies.
AU Durbin A P; Cho C J; Elkins W R; Wyatt L S; Moss B; Murphy B R
CS Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0720, USA.. adurbin@nih.gov
SO JOURNAL OF INFECTIOUS DISEASES, (1999 Jun) 179 (6) 1345-51.
Journal code: IH3. ISSN: 0022-1899.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 199908
EW 19990804
AB Two parainfluenza **virus** type 3 (PIV3) vaccine candidates-cp45, a live attenuated derivative of the JS wild type (wt), and a replication-defective vaccinia **virus recombinant** expressing the hemagglutinin-neuraminidase or fusion glycoprotein of human PIV3 (modified vaccinia **virus Ankara [MVA**]/PIV3 recombinants)-were evaluated in rhesus monkeys to determine whether successful immunization could be achieved in the presence of passively transferred PIV3 antibodies. The cp45 **virus**, administered intranasally (in) and intratracheally (it) in the presence of high levels of PIV3 antibodies, replicated efficiently in the nasopharynx and protected against challenge with wt human PIV3. The **MVA** recombinants, administered in, it, and intramuscularly in the absence of passive antibody, conferred protection against replication of PIV3 wt challenge **virus**, but this was largely abrogated when immunization occurred in the presence of passive antibodies. Because immunization for the prevention of HPIV3 disease must occur in early infancy when maternal antibodies are present, the live attenuated cp45

virus continues to be a promising vaccine candidate for this age group.

L2 ANSWER 12 OF 63 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 7
AN 2000:40012 CAPLUS
TI New prospects for the development of a vaccine against human immunodeficiency **virus** type 1. An overview
AU Girard, Marc; Habel, Andre; Chanel, Chantal
CS Unite de virologie moleculaire, departement de virologie. Institut Pasteur, Paris, 75015, Fr.
SO C. R. Acad. Sci., Ser. III (1999), 322(11), 959-966
CODEN: CRASEV; ISSN: 0764-4469
PB Editions Scientifiques et Medicales Elsevier
DT Journal
LA English
AB During the past few years, definite progress has been made in the field of human immunodeficiency **virus** type 1 (HIV-1) vaccines. Initial attempts using envelope gp 120 or gp 140 from T-cell line-adapted (TCLA) HIV-1 strains to vaccinate chimpanzees showed that neutralizing antibody-based immune responses were protective against challenge with homologous TCLA **virus** strains or strains with low replicative capacity, but these neutralizing antibodies remained inactive when tested on primary HIV-1 isolates, casting doubts on the efficacy of gp120-based vaccines in the natural setting. Development of a live attenuated simian immunodeficiency **virus** (SIV) vaccine was undertaken in the macaque model using whole live SIV bearing multiple deletions in the nef, vpr and vpx genes. This vaccine provided remarkable protective efficacy against wild-type SIV challenge, but the deletion mutants remain pathogenic, notably in neonate monkeys. Study of the mechanisms of protection in the SIV model unravelled the importance of the T-cell responses, whether in the form of cytotoxic T-lymphocyte (CTL) killing activity, or in that of antiviral factor secretion of cytokines, .beta.-chemokines and other unidentified antiviral factors by CD8+ T-cells. Induction of such a response is being sought at this time using various live **recombinant virus** vaccines, either poxvirus or alphavirus vectors or DNA vectors, which can be combined together or with a gp120/gp140 boost in various prime-boost combination strategies. New vectors include attenuated vaccinia **virus** NYVAC, modified vaccinia strain **Ankara** (**MVA**), Semliki Forest **virus**, Venezuelan equine encephalitis **virus**, and Salmonellas. Recent DNA prime-poxvirus boost combination regimens have generated promising protection results against SIV or SIV/HIV (SHIV) challenge in macaque models. Emphasis is also put on the induction of a mucosal immune response, involving both a secretory IgA response and a mucosal CTL response which could constitute a "first line of defense" in the vaccinated host. Finally, a totally novel vaccine approach based on the use of Tat or Tat and Rev antigens has been shown to induce efficient protection from challenge with pathogenic SIV or SHIV in vaccinated macaques. The only vaccine in phase 3 clin. trials in human volunteers is a gp120-based vaccine, AIDSVAX. A prime-boost combination of a **recombinant** canarypoxvirus and a subunit gp120 vaccine is in phase 2. Emphasis has been put recently on the necessity of testing prototype vaccines in developing countries using immunogens derived from local **virus** strains. Trial sites have thus been identified in Kenya, Uganda, Thailand and South Africa where phase I trials have begun or are expected to start presently.

L2 ANSWER 13 OF 63 MEDLINE DUPLICATE 8
AN 2000016513 MEDLINE
DN 20016513
TI Gene gun intradermal DNA immunization followed by boosting with modified vaccinia **virus Ankara**: enhanced CD8+ T cell immunogenicity and protective efficacy in the influenza and malaria models.
AU Degano P; Schneider J; Hannan C M; Gilbert S C; Hill A V
CS PowderJect Pharmaceuticals plc, 4 Robert Robinson Avenue, The Oxford

Science Park, Oxford, UK.. pilar.degano@ndm.ox.ac.uk
SO VACCINE, (1999 Nov 12) 18 (7-8) 623-32.
Journal code: X60. ISSN: 0264-410X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200004
EW 20000401
AB In influenza and malaria, CD8+ T cells play an important role in protective immunity in mice. An immunization strategy consisting of DNA priming followed by boosting with **recombinant** modified vaccinia **virus Ankara** (**MVA**) induces complete protection, associated with high levels of CD8+ T cells, against Plasmodium berghei sporozoite challenge in mice. Intradermal delivery of DNA with a gene gun requires smaller amounts of DNA than intramuscular injection, in order to induce similar levels of immune responses. The present study compares both routes for the induction of specific CD8+ T cell responses and protection using different prime-boost immunization regimes in the influenza and the malaria models. In the DNA/**MVA** regime, equally high CD8+ T cell responses and levels of protection are achieved using ten times less DNA when delivered with a gene gun compared to intramuscular injection.

L2 ANSWER 14 OF 63 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 9
AN 1999054991 EMBASE
TI Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime.
AU Hanke T.; Neumann V.C.; Blanchard T.J.; Sweeney P.; Hill A.V.S.; Smith G.L.; McMichael A.
CS T. Hanke, Molecular Immunology Group, Institute of Molecular Medicine, University of Oxford, Headley Way, Headington, Oxford OX3 9DS, United Kingdom. hanke@ermine.ox.ac.uk
SO Vaccine, (12 Feb 1999) 17/6 (589-596).
Refs: 48
ISSN: 0264-410X CODEN: VACCDE
PUI S 0264-410X(98)00238-2
CY United Kingdom
DT Journal; Article
FS 022 Human Genetics
026 Immunology, Serology and Transplantation
037 Drug Literature Index
LA English
SL English
AB Reliable and effective induction of T-lymphocytes (CTL) is one of the prime objectives of vaccine research. Previously, novel HIV vaccine candidates were constructed as a string of CTL epitopes (20 human, 3 macaque and 1 mouse) delivered using a DNA vector [Hanke T, Schneider J, Gilbert SG, Hill AVS, McMichael A. DNA multi-CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice. Vaccine 1998;16:426-435.] or modified vaccinia **Ankara** (**MVA** [Hanke T, Blanchard TJ, Schneider J, Ogg GS, Tan R, Becker MSC, Gilbert SG, Hill AVS, Smith GL, McMichael A. Immunogenicities of intravenous and intramuscular administrations of **MVA**-based multi-CTL epitope vaccine for HIV in mice. J Gen Virol 1998;79:83-90.]), i.e. vaccine vehicles acceptable for use in humans. In mice, a single intramuscular (i.m.) needle injection of either vaccine alone elicited good CTL responses. Here, it is demonstrated that the multi-epitope DNA also induced CTL when delivered intradermally using the Accell.RTM. gene gun. The CTL responses increased after re-immunization and after three deliveries were comparable to those induced by a single i.m. injection. Recent evidence indicates that combining routes and vaccine vehicles enhances the immunogenicity of vaccine-delivered or - encoded antigens. Here, it is shown that administration of DNA by an i.m. priming/gene gun boosting more efficiently induced CTL than gene gun priming/i.m. boosting. A similar increment was obtained by sequential vaccinations using a gene gun-delivered DNA followed by **recombinant MVA**. Thus

particular sequences of routes or vaccine vehicles rather than simple prime- boost delivery of a single vaccine is critical for an effective elicitation of CTL.

L2 ANSWER 15 OF 63 MEDLINE DUPLICATE 10
AN 1999180116 MEDLINE
DN 99180116
TI Gene transfer in astrocytes: comparison between different delivering methods and expression of the HIV-1 protein Nef.
AU Ambrosini E; Ceccherini-Silberstein F; Erfle V; Aloisi F; Levi G
CS Laboratory of Organ and System Pathophysiology, Istituto Superiore di Sanit`a, Rome, Italy.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1999 Mar 1) 55 (5) 569-77.
Journal code: KAC. ISSN: 0360-4012.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199907
EW 19990705
AB To identify a good system to introduce foreign genes into normal and tumoral astrocytes, we studied the efficiency of two chemical methods, calcium phosphate precipitation and lipofection, and of a viral-mediated transfer by a vector derived from the highly attenuated modified vaccinia **virus Ankara** (**MVA**). Using the beta-galactosidase (beta-gal) gene (lacZ) as reporter, we searched for optimal experimental conditions to obtain an efficient gene transfer into human embryonic and neonatal rat astrocytes and into a human astrocytoma cell line (U373 MG). The beta-gal protein production was evaluated by cytochemical staining and enzymatic activity assay. Among chemical methods, lipofection was the most efficient system to transfect astrocytes in providing up to 60% of beta-gal-positive cells in all the cell types analyzed. **MVA** infection also proved to be an efficient system to introduce heterologous genes into human embryonic astrocytes that appeared 80-100% positive 48-96 hr after an infection at a multiplicity of 1-10. In contrast, only a limited infection was observed with rat astrocytes, human astrocytoma cells, and human leptomeningeal cells. A **recombinant** **MVA** vector expressing the human immunodeficiency **virus**-1 (HIV-1) regulatory protein Nef was used to transfect human embryonic astrocytes, and the resulting Nef expression was compared with that detected after lipofection in the same cells. By Western blot analysis, Nef expression was observed in human astrocytes 24-96 hr after infection and was similar to that present in stably HIV-1-infected astrocytoma cells. Lipofection resulted in lower Nef expression. In spite of these promising results, the negative effects of **MVA** infection on cell viability and the possibility that a productive infection occurs in human embryonic astrocytes limit the use of this vector for gene delivery in developmentally immature human glial cells.

L2 ANSWER 16 OF 63 MEDLINE DUPLICATE 11
AN 1999451172 MEDLINE
DN 99451172
TI Priming and boosting immunity to respiratory syncytial **virus** by **recombinant** replication-defective vaccinia **virus** **MVA**.
AU Wyatt L S; Whitehead S S; Venanzi K A; Murphy B R; Moss B
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Drive MSC 0445, Bethesda, MD 20892-0445, USA.
SO VACCINE, (1999 Oct 14) 18 (5-6) 392-7.
Journal code: X6O. ISSN: 0264-410X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200003
EW 20000303

AB Intranasal and intramuscular immunizations of mice with the highly attenuated **MVA** strain of vaccinia **virus** expressing the respiratory syncytial **virus** (RSV) F or G glycoprotein induced higher RSV antibody titers than those achieved by infection with RSV and greatly restricted the replication of RS challenge **virus** in both the upper and lower respiratory tracts. In addition, a **recombinant MVA** expressing both RSV F and G was stable and was as immunogenic as a combination of two single **recombinant** viruses. The levels of antibodies to RSV F and G, induced by previous intranasal infection with attenuated RSV, were boosted by intramuscular immunization with **recombinant MVA**. These data support further development of **recombinant MVA** as a RSV vaccine.

L2 ANSWER 17 OF 63 MEDLINE
AN 1999237843 MEDLINE
DN 99237843
TI Ty **virus**-like particles, DNA vaccines and Modified Vaccinia **Virus Ankara**; comparisons and combinations.
AU Gilbert S C; Schneider J; Plebanski M; Hannan C M; Blanchard T J; Smith G L; Hill A V
CS Wellcome Trust Centre for Human Genetics, University of Oxford, UK.
SO BIOLOGICAL CHEMISTRY, (1999 Mar) 380 (3) 299-303.
Journal code: CK4. ISSN: 1431-6730.
CY GERMANY: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199909
EW 19990901
AB Three types of vaccine, all expressing the same antigen from Plasmodium berghei, or a CD8+ T cell epitope from that antigen, were compared for their ability to induce CD8+ T cell responses in mice. Higher levels of lysis and numbers of IFN-gamma secreting T cells were primed with Ty **virus**-like particles and Modified Vaccinia **Virus Ankara** (**MVA**) than with DNA vaccines, but none of the vaccines were able to protect immunised mice from infectious challenge even after repeated doses. However, when the immune response was primed with one type of vaccine (Ty-VLPs or DNA) and boosted with another (**MVA**) complete protection against infection was achieved. Protection correlated with very high levels of IFN-gamma secreting T cells and lysis. This method of vaccination uses delivery systems and routes that can be used in humans and could provide a generally applicable regime for the induction of high levels of CD8+ T cells.

L2 ANSWER 18 OF 63 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.
AN 1998412732 EMBASE
TI Protection against lethal Japanese encephalitis **virus** infection of mice by immunization with the highly attenuated **MVA** strain of vaccinia **virus** expressing JEV prM and E genes.
AU Nam J.-H.; Wyatt L.S.; Chae S.-L.; Cho H.-W.; Park Y.-K.; Moss B.
CS B. Moss, 4 Center Drive MSC 0445, Bethesda, MD 20892-0445, United States. bmoss@nih.gov
SO Vaccine, (21 Jan 1999) 17/3 (261-268).
Refs: 30
ISSN: 0264-410X CODEN: VACCDE
PUI S 0264-410X(98)00156-X
CY United Kingdom
DT Journal; Article
FS 004 Microbiology
026 Immunology, Serology and Transplantation
037 Drug Literature Index
LA English
SL English
AB Genes encoding the glycosylated precursor of the membrane (prM) and envelope (E) proteins of a Korean strain of Japanese encephalitis **virus** (JEV) were inserted into the genome of the host-range

restricted, highly attenuated, and safety-tested **MVA** strain of vaccinia **virus**. **MVA** recombinants containing the JEV genes, under strong synthetic or modified H5 vaccinia **virus** promoters, were isolated. Synthesis of JEV prM and E proteins was detected by immunofluorescence microscopy, flow cytometry, and polyacrylamide gel electrophoresis. Mice inoculated and boosted by various routes with either of the **MVA** recombinants produced JEV neutralizing antibodies, that had titres comparable with those induced by an inactivated JEV vaccine, as well as haemagglutination-inhibiting antibodies. Mice immunized with 2x 106 infectious units of **MVA**/JEV recombinants by intramuscular or intraperitoneal routes were completely protected against a 105 LD50 JEV challenge at 9 weeks of age.

L2 ANSWER 19 OF 63 SCISEARCH COPYRIGHT 2000 ISI (R)
AN 1999:825620 SCISEARCH
GA The Genuine Article (R) Number: 249GZ
TI Approaches to improve engineered vaccines for human immunodeficiency **virus** and other viruses that cause chronic infections
AU Berzofsky J A (Reprint); Ahlers J D; Derby M A; Pendleton C D; Arichi T; Belyakov I M
CS NCI, MOL IMMUNOGENET & VACCINE RES SECT, METAB BRANCH, NIH, BLDG 10, ROOM 6B-12, MSC 1578, BETHESDA, MD 20892 (Reprint)
CYA USA
SO IMMUNOLOGICAL REVIEWS, (AUG 1999) Vol. 170, pp. 151-172.
Publisher: MUNKSGAARD INT PUBL LTD, 35 NORRE SOGADE, PO BOX 2148, DK-1016 COPENHAGEN, DENMARK.
ISSN: 0105-2896.
DT General Review; Journal
FS LIFE
LA English
REC Reference Count: 172
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB We used several approaches to develop enhanced vaccines for chronic viral infections such as human immunodeficiency **virus** (HIV) and hepatitis C **virus** (HCV). 1) Selected epitopes were used to avoid potentially harmful immune responses. 2) Linkage between helper and cytotoxic T-lymphocyte (CTL) epitopes was found to be important. 3) We developed an ''epitope enhancement'' approach modifying the sequences of epitopes to make more potent vaccines, including examples for HIV and HCV epitopes presented by murine class II and human class I major histocompatibility complex (MHC) molecules. 4) CTL avidity was found to be important for clearing viral infections in vivo, and the mechanism was examined. High-avidity CTLs, however, were found to undergo apoptosis when confronted with high-density antigen, through a mechanism involving tumor necrosis factor (TNF), TNF-RII, and a permissive state induced through the T-cell receptor. 5) We employed cytokines in the adjuvant to steer immune responses toward desired phenotypes, and showed synergy between cytokines. 6) Intrarectal immunization with peptide vaccine induced mucosal and systemic CTL. Local mucosal CTL were found to be critical for resistance to mucosal viral transmission and this resistance was enhanced with mucosally delivered interleukin-12. 7) We used an asymmetry in induction of mucosal and systemic immune responses to circumvent pre-existing vaccinia immunity for use of **recombinant** vaccinia vaccines.

L2 ANSWER 20 OF 63 MEDLINE DUPLICATE 12
AN 2000032581 MEDLINE
DN 20032581
TI Cytotoxic T lymphocytes specific for the simian immunodeficiency **virus**.
AU Letvin N L; Schmitz J E; Jordan H L; Seth A; Hirsch V M; Reimann K A; Kuroda M J
CS Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA.. nletvin@caregroup.harvard.edu
SO IMMUNOLOGICAL REVIEWS, (1999 Aug) 170 127-34. Ref: 33
Journal code: GG4. ISSN: 0105-2896.
CY Denmark
DT Journal; Article; (JOURNAL ARTICLE)

General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Priority Journals
EM 200002
EW 20000204
AB A non-human primate model for acquired immunodeficiency syndrome (AIDS), the simian immunodeficiency **virus** (SIV)-infected rhesus monkey, was used to explore the role of the AIDS **virus**-specific cytotoxic T-lymphocyte (CTL) response in disease pathogenesis. This CTL response was measured using the major histocompatibility complex (MHC) class I/peptide tetramer technology. Large numbers of tetramer-binding CD8+ T lymphocytes were demonstrable not only in the peripheral blood, but in lymph nodes and even in semen of chronically SIV-infected monkeys. The central role of these effector T lymphocytes in containing SIV spread during primary infection was demonstrated by showing that early SIV clearance during primary infection correlated with the emergence of the tetramer binding CD8+ T lymphocytes and that in vivo depletion of CD8+ lymphocytes eliminated the ability of the infected monkeys to contain SIV replication. These observations suggest that an effective AIDS vaccine should elicit a potent **virus**-specific CTL response. In fact, a live, **recombinant** SIV vaccine constructed using the attenuated pox **virus** vector modified vaccinia **Ankara** (**MVA**) elicited a high-frequency CTL response, comparable in magnitude to that elicited by SIV infection itself. This suggests that vaccine modalities such as **MVA** may prove useful in creating an effective human immunodeficiency **virus** (HIV) vaccine. These studies also indicate the power of both the SIV/macaque model and MHC class I/peptide tetramers for assessing AIDS vaccine strategies.

L2 ANSWER 21 OF 63 AIDSLINE
AN 1998:22110 AIDSLINE
DN AIDS-98930426
TI Induction of mucosal CTL response by intra-rectal immunization with a replication-deficient **recombinant** vaccinia **virus** expressing HIV 89.6 env protein.
AU Belyakov I M; Wyatt L S; Earl P; Moss B; Berzofsky J A
CS National Cancer Institute, National Institutes of Health, Bethesda, MD.
SO HIV Pathog Treat Conf, (1998). pp. 81 (Abstract No. 4003).
CY United States
DT (MEETING ABSTRACTS)
FS AIDS
LA English
EM 199812
AB To reduce the risk of vaccine **virus** dissemination, **MVA** (modified vaccinia **virus Ankara**) was derived for use as a smallpox vaccine by repeated passaging in chicken embryo fibroblasts. Six major deletions in the genome, resulting in the loss of 30,000 base pairs, made **MVA** replication deficient in most mammalian cell lines, BALB/c mice were intrarectally (IR) immunized with **recombinant MVA** expressing the env gene of HIV-1 strain 89.6. As a control we used a wild type **recombinant** vaccinia **virus** (vBD3) expressing the same protein. A single mucosal immunization with either strain of **recombinant** vaccinia **virus** (**MVA** 89.6 or vBD3) was able to elicit antigen-specific mucosal and systemic CTL responses. In mucosal sites, vBD3 **virus** was able to induce a Peyer's patch (PP) CTL response only, whereas **recombinant MVA** elicited both HIV-specific PP and lamina propria CTL responses. The level of the PP CTL responses after IR immunization with a replication-deficient **recombinant** vaccinia **virus** was significantly higher than after mucosal immunization with wild type **recombinant** **virus**. Intraperitoneal immunization induced antigen-specific CTL responses in PP only after inoculation with **recombinant** **MVA virus**, not vBD3 **virus**. 89.6-specific CTL

crossreacted with the corresponding epitope from HIV-1 MN and not that from HIV-1 IIIB. This replication-deficient **recombinant** modified vaccinia **virus** may be safer and at least as effective for mucosal immunization as conventional **recombinant** vaccinia **virus**.

L2 ANSWER 22 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1999:8123 CAPLUS
DN 130:61998
TI Methods for vaccination which generate a CD8+ T cell immune response using CTL (cytotoxic T lymphocyte) response epitopes with utility in primates
IN Mcmichael, Andrew James; Hill, Adrian Vivian Sinton; Gilbert, Sarah Catherine; Schneider, Joerg; Plebanski, Magdalena; Hanke, Tomas; Smith, Geoffrey Lilley; Blanchard, Tom
PA Isis Innovation Limited, UK
SO PCT Int. Appl., 87 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9856919	A2	19981217	WO 1998-GB1681	19980609
WO 9856919	A3	19990617		

W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG

AU 9880266	A1	19981230	AU 1998-80266	19980609
EP 979284	A2	20000216	EP 1998-928434	19980609

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, NL, SE, IE, FI

PRAI GB 1997-11957 19970609
WO 1998-GB1681 19980609

AB New methods and reagents for vaccination are described which generate a CD8 T cell immune response against malarial and other antigens such as viral and tumor antigens. These studies have provided clear evidence that a novel sequential immunization regime employing a non-replicating pox **virus** as a boost is capable of inducing a strong protective CD8+ T cell response against the malaria parasite. The crit. step in this vaccination regimen is the use of non-replicating or replication-impaired **recombinant** poxviruses to boost a pre-existing cytotoxic T lymphocyte response. This response can be primed using different antigen delivery systems such as a DNA vaccine, a **recombinant** Ty **virus**-like particle, a **recombinant** adenovirus and irradiated sporozoites. Importantly, this immunization regiment is also demonstrated to be highly immunogenic for CD8+ T cells in primates. In factstron SIV-gag-specific cytotoxic T lymphocyte response were induced in 3 out of 3 macaques with both plasmid DNA and **MVA** expressing epitope strings. Higher primates such as chimpanzees were also protected in the same way.

L2 ANSWER 23 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1998:210865 CAPLUS
DN 128:253806
TI **Recombinant** modified vaccinia **virus Ankara** (**MVA**) expressing dengue **virus** antigens, and the use thereof in vaccines
IN Cardosa, Mary Jane; Sutter, Gerd; Erfle, Volker
PA Bavarian Nordic Research Institute A/S, Den.; University Malaysia Sarawak; Gsf - Forschungszentrum fur Umwelt und Gesundheit; Cardosa, Mary Jane; Sutter, Gerd; Erfle, Volker
SO PCT Int. Appl., 23 pp.
CODEN: PIXXD2

DT Patent
LA English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 9813500	A2	19980402	WO 1997-EP5214	19970923
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
AU 9745565	A1	19980417	AU 1997-45565	19970923
EP 951555	A2	19991027	EP 1997-943887	19970923
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				

PRAI DK 1996-1035 19960924
WO 1997-EP5214 19970923

AB **Recombinant MVA** viruses contg. and/or capable of expressing dengue **virus** antigens and the use of such **recombinant MVA** for vaccination are claimed. The antigens correspond to the dengue glycoproteins E, preE, and NS1. Thus, vaccines can be prepd. from **recombinant MVA** encoding antigens for all dengue serotypes (1-4). **MVA** vectors also contain vaccinia **virus** early/late promoter P7.5 and encode phage T7 RNA polymerase. The vaccine can be used for the treatment or prevention of dengue **virus** infection.

L2 ANSWER 24 OF 63 MEDLINE DUPLICATE 13
AN 1998374314 MEDLINE
DN 98374314
TI **Recombinant** modified vaccinia **virus Ankara** -simian immunodeficiency **virus** gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer.
AU Seth A; Ourmanov I; Kuroda M J; Schmitz J E; Carroll M W; Wyatt L S; Moss B; Forman M A; Hirsch V M; Letvin N L
CS Harvard Medical School, Division of Viral Pathogenesis, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA.
NC AI35166 (NIAID)
AI26507 (NIAID)
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 Aug 18) 95 (17) 10112-6.
Journal code: PV3. ISSN: 0027-8424.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199811
EW 19981103
AB The utility of modified vaccinia **virus Ankara** (**MVA**) as a vector for eliciting AIDS **virus**-specific cytotoxic T lymphocytes (CTL) was explored in the simian immunodeficiency **virus** (SIV)/rhesus monkey model. After two intramuscular immunizations with **recombinant MVA**-SIVSM gag pol, the monkeys developed a Gag epitope-specific CTL response readily detected in peripheral blood lymphocytes by using a functional killing assay. Moreover, those immunizations also elicited a population of CD8+ T lymphocytes in the peripheral blood that bound a specific major histocompatibility complex class I/peptide tetramer. These Gag epitope-specific CD8+ T lymphocytes also were demonstrated by using both functional and tetramer-binding assays in lymph nodes of the immunized monkeys. These observations suggest that **MVA** may prove a useful vector for an HIV-1 vaccine. They also suggest that tetramer staining may

be a useful technology for monitoring CTL generation in vaccine trials in nonhuman primates and in humans.

L2 ANSWER 25 OF 63 MEDLINE DUPLICATE 14
AN 1998406234 MEDLINE
DN 98406234
TI Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient **recombinant** vaccinia **virus** expressing human immunodeficiency **virus** 89.6 envelope protein.
AU Belyakov I M; Wyatt L S; Ahlers J D; Earl P; Pendleton C D; Kelsall B L; Strober W; Moss B; Berzofsky J A
CS Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.
SO JOURNAL OF VIROLOGY, (1998 Oct) 72 (10) 8264-72.
Journal code: KCV. ISSN: 0022-538X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199812
EW 19981202
AB To improve the safety of **recombinant** vaccinia **virus** vaccines, modified vaccinia **virus Ankara** (**MVA**) has been employed, because it has a replication defect in most mammalian cells. Here we apply **MVA** to human immunodeficiency **virus** type 1 (HIV-1) vaccine development by incorporating the envelope protein gp160 of HIV-1 primary isolate strain 89.6 (**MVA** 89.6) and use it to induce mucosal cytotoxic-T-lymphocyte (CTL) immunity. In initial studies to define a dominant CTL epitope for HIV-1 89.6 gp160, we mapped the epitope to a sequence, IGPGRAFYAR (from the V3 loop), homologous to that recognized by HIV MN loop-specific CTL and showed that HIV-1 MN-specific CTLs cross-reactively recognize the corresponding epitope from strain 89.6 presented by H-2Dd. Having defined the CTL specificity, we immunized BALB/c mice intrarectally with **recombinant MVA** 89.6. A single mucosal immunization with **MVA** 89.6 was able to elicit long-lasting antigen-specific mucosal (Peyer's patch and lamina propria) and systemic (spleen) CTL responses as effective as or more effective than those of a replication-competent vaccinia **virus** expressing 89.6 gp160. Immunization with **MVA** 89.6 led to (i) the loading of antigen-presenting cells in vivo, as measured by the ex vivo active presentation of the P18-89.6 peptide to an antigen-specific CTL line, and (ii) the significant production of the proinflammatory cytokines (interleukin-6 and tumor necrosis factor alpha) in the mucosal sites. These results indicate that nonreplicating **recombinant** **MVA** may be at least as effective for mucosal immunization as replicating **recombinant** vaccinia **virus**.

L2 ANSWER 26 OF 63 MEDLINE DUPLICATE 15
AN 1999077187 MEDLINE
DN 99077187
TI Protection from Plasmodium berghei infection by priming and boosting T cells to a single class I-restricted epitope with **recombinant** carriers suitable for human use.
AU Plebanski M; Gilbert S C; Schneider J; Hannan C M; Layton G; Blanchard T; Becker M; Smith G; Butcher G; Sinden R E; Hill A V
CS Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, GB..
mplebans@worf.molbiol.ox.ac.uk
SO EUROPEAN JOURNAL OF IMMUNOLOGY, (1998 Dec) 28 (12) 4345-55.
Journal code: EN5. ISSN: 0014-2980.
CY GERMANY: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals

EM 199903
EW 19990301
AB The desirability of inducing cytotoxic T cell responses to defined epitopes in humans has led to the development of a variety of **recombinant** delivery systems. **Recombinant** protein particles derived from a yeast retrotransposon (Ty) and the modified **Ankara** vaccinia (**MVA**) **virus** can deliver large epitope strings or even whole proteins. Both have previously been administered safely in humans. Immunization with **recombinant** Ty and **MVA** containing a single Plasmodium berghei class I-binding epitope provided 95% sterile protection against malaria in mice. The sequence of immunization, Ty followed by **MVA**, was critical to elicit high levels of IFN-gamma-producing cells and protection. The reciprocal sequence (**MVA**/TY) or homologous boosting was not protective. Both constructs (Ty and **MVA**) contain the H-2Kd-restricted pb9 CTL epitope from the circumsporozoite protein of P. berghei among a string of 8-15 human P. falciparum-derived CTL epitopes restricted through 7 common HLA alleles as well as widely recognized CD4 T cell epitopes. Thus, the novel **recombinant** Ty/**MVA** prime/boost combination with these constructs provides a safe alternative for evaluation for human vaccination against P. falciparum malaria.

L2 ANSWER 27 OF 63 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 16
AN 1998224768 EMBASE
TI The immunogenicity and efficacy of intranasally or parenterally administered replication-deficient vaccinia-parainfluenza **virus** type 3 recombinants in rhesus monkeys.
AU Durbin A.P.; Wyatt L.S.; Slew J.; Moss B.; Murphy B.R.
CS A.P. Durbin, Laboratory of Infectious Diseases, Natl. Inst. of Allergy/Infect. Dis., National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, United States
SO Vaccine, (1998) 16/13 (1324-1330).
Refs: 35
ISSN: 0264-410X CODEN: VACCDE
PUI S 0264-410X(98)00010-3
CY United Kingdom
DT Journal; Article
FS 004 Microbiology
026 Immunology, Serology and Transplantation
037 Drug Literature Index
LA English
SL English
AB Immunization of rhesus monkeys with modified vaccinia **Ankara** (**MVA**) recombinants expressing the haemagglutinin-neuraminidase (HN) or fusion (F) glycoproteins of human parainfluenza **virus** type 3 (HPIV3) was compared with an intranasallyadministered live, attenuated HPIV3 vaccine candidate, the cp45 derivative of the JS strain of wildtype HPIV3. The **MVA** recombinants, when given parenterally (i.m.) or as a parenteral-local (i.m. and i.t.) combination, induced an antibody response comparable to that of cp45 and protected the upper and lower respiratory tracts of the rhesus monkeys against challenge with wildtype HPIV3. When given by the i.n. route alone, the **MVA**/PIV3 recombinants induced a serum antibody response that was comparable to that of cp45 and induced resistance in the lower respiratory tract. Despite the ability of the intranasally-administered **MVA**/PIV3 recombinants to stimulate a good serological response and to protect the lower respiratory tract, they unexpectedly failed to induce a significant level of resistance in the upper respiratory tract. The live, attenuated **virus** vaccine candidate induced almost complete resistance in both the upper and lower tracts. The data thus identify two vaccine candidates that can protect both the upper and lower respiratory tracts of rhesus monkey, parenterally-administered **MVA**/PIV3 and intranasally-administered cp45. Further studies with these vaccines in non-human primates and humans should identify the relative merits of these immunogens for use in the very young infant.

L2 ANSWER 28 OF 63 MEDLINE DUPLICATE 17
AN 1998390100 MEDLINE
DN 98390100
TI Effect of 3-beta-hydroxysteroid dehydrogenase gene deletion on virulence and immunogenicity of different vaccinia viruses and their recombinants.
AU Sroller V; Kutinova L; Nemeckova S; Simonova V; Vonka V
CS Institute of Hematology and Blood Transfusion, Praha, Czech Republic.
SO ARCHIVES OF VIROLOGY, (1998) 143 (7) 1311-20.
Journal code: 8L7. ISSN: 0304-8608.
CY Austria
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199811
EW 19981103
AB 3-beta-Hydroxysteroid dehydrogenase (3-beta-HSD) activity coded for by the A44L gene of vaccinia **virus** (VV) was demonstrated in CV-1 cultures infected by all five VV strains tested, viz. WR, Praha **virus**, DRYVAX Wyeth-derived **virus** (DD), LIVP and **MVA**. Deletion of the A44L gene in two Praha **virus** -derived clones (the moderately virulent P13 and the highly attenuated P20), the WR and DD viruses resulted in absence of 3-beta-HSD activity from infected cultures. The virulence for mice of P13 was not affected, and that of WR was only slightly decreased, by the A44L gene deletion. **Recombinant** VVs expressing either varicella-zoster **virus** glycoprotein E (VZV-gE) or hepatitis B **virus** preS2-S protein (HBV-preS2-S) and their respective A44L deleted mutants were used in immunogenicity tests in mice. In terms of antibody response to VV and the **recombinant** proteins, the deletion resulted in a lowering the immunogenicity in the moderately virulent clone P13 **virus** and its progenies. In the highly attenuated P20 and DD viruses and their progenies no effects were apparent.

L2 ANSWER 29 OF 63 MEDLINE DUPLICATE 18
AN 1998264493 MEDLINE
DN 98264493
TI Modified vaccinia **virus Ankara** undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine.
AU Blanchard T J; Alcami A; Andrea P; Smith G L
CS Sir William Dunn School of Pathology, University of Oxford, UK.
SO JOURNAL OF GENERAL VIROLOGY, (1998 May) 79 (Pt 5) 1159-67.
Journal code: I9B. ISSN: 0022-1317.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
OS GENBANK-AF016273
EM 199808
EW 19980803
AB Modified **virus Ankara** (**MVA**) is a vaccinia **virus** (VV) strain that was attenuated by serial passage through chick embryo fibroblasts (CEFs) and contains six large genomic deletions compared with parental **virus**. **MVA** replicates well in CEFs, but poorly in most mammalian cells. **Recombinant** **MVA** is a promising human vaccine candidate due to its restricted host range, immunogenicity and avirulence in animal models, and excellent safety record as a smallpox vaccine. Here we present a further characterization of **MVA** and demonstrate that: (i) **MVA** can replicate, albeit poorly, in transformed human cell lines, but not in primary human fibroblasts although there is limited cell-to-cell spread; (ii) **MVA** is a potent inducer of type I interferon (IFN) from primary human cells, which may restrict **virus** spread in vivo; and (iii) unlike other VV strains, **MVA** does not express soluble receptors for IFN-gamma, IFN-alpha/beta, tumour necrosis factor and CC chemokines, but does express a soluble interleukin-1beta receptor. This

provides a plausible and testable explanation for the good immunogenicity of **MVA** despite its poor replication in mammals. The implications of these findings for the use of **MVA** as a safe and immunogenic human vaccine candidate are discussed.

L2 ANSWER 30 OF 63 MEDLINE DUPLICATE 19
AN 1998232260 MEDLINE
DN 98232260
TI Transient marker stabilisation: a general procedure to construct marker-free **recombinant** vaccinia **virus**.
AU Scheiflinger F; Dorner F; Falkner F G
CS Immuno AG, Orth/Donau, Austria.
SO ARCHIVES OF VIROLOGY, (1998) 143 (3) 467-74.
Journal code: 8L7. ISSN: 0304-8608.
CY Austria
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199807
EW 19980704
AB **Recombinant** vaccinia viruses based on the highly attenuated Modified Vaccinia **Ankara** (**MVA**) strain expressing HIV-1 antigen genes were constructed by a novel procedure involving the transient use of two marker genes. The selectable markers used, the Escherichia coli guanine phosphoribosyltransferase (gpt) and the beta-galactosidase (lacZ) genes, are not retained within the final **recombinant virus**. The transient marker stabilisation (TMS) procedure allows the generation of marker-free **recombinant** viruses in a series of simple plaque purification steps. HIV-1 gag pol genes were inserted into two loci of vaccinia **MVA** demonstrating the efficiency of the procedure.

L2 ANSWER 31 OF 63 MEDLINE DUPLICATE 20
AN 1998206860 MEDLINE
DN 98206860
TI Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia **virus Ankara**.
AU Schneider J; Gilbert S C; Blanchard T J; Hanke T; Robson K J; Hannan C M; Becker M; Sinden R; Smith G L; Hill A V
CS Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, UK..
joerg.schneider@ndm.ox.ac.uk
SO NATURE MEDICINE, (1998 Apr) 4 (4) 397-402.
Journal code: CG5. ISSN: 1078-8956.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199807
EW 19980701
AB Immunization with irradiated sporozoites can protect against malaria infection and intensive efforts are aimed at reproducing this effect with subunit vaccines. A particular sequence of subunit immunization with pre-erythrocytic antigens of Plasmodium berghei, consisting of single dose priming with plasmid DNA followed by a single boost with a **recombinant** modified vaccinia **virus Ankara** (**MVA**) expressing the same antigen, induced unprecedented complete protection against P. berghei sporozoite challenge in two strains of mice. Protection was associated with very high levels of splenic peptide-specific interferon-gamma-secreting CD8+ T cells and was abrogated when the order of immunization was reversed. DNA priming followed by **MVA** boosting may provide a general immunization regime for induction of high levels of CD8+ T cells.

L2 ANSWER 32 OF 63 MEDLINE DUPLICATE 21

AN 1999114127 MEDLINE
DN 99114127
TI Gene transfer into human dendritic antigen-presenting cells by vaccinia **virus** and adenovirus vectors.
AU Di Nicola M; Siena S; Bregni M; Longoni P; Magni M; Milanesi M; Matteucci P; Mortarini R; Anichini A; Parmiani G; Drexler I; Erfle V; Sutter G; Gianni A M
CS Division of Medical Oncology (OMC), Istituto Nazionale Tumori, Milan, Italy.
SO CANCER GENE THERAPY, (1998 Nov-Dec) 5 (6) 350-6.
Journal code: CE3. ISSN: 0929-1903.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199905
EW 19990504
AB In a search for means to deliver exogenous gene(s) into human dendritic cells (DCs) from the perspective of tumor-specific vaccination, we have evaluated two **recombinant** viruses, both of which carry a reporter gene which is namely a modified vaccinia **virus Ankara** (**MVA**) and an adenovirus, as possible expression vectors. The **recombinant MVA**-P11 LZ vector carries the Escherichia coli lacZ gene coding for the enzyme beta-galactosidase, and the **recombinant** Ad-MFG-AP vector carries a modified membrane-exposed alkaline phosphatase (AP) gene. DCs were generated ex vivo in the presence of tumor necrosis factor-alpha, granulocyte macrophage colony-stimulating factor, stem cell factor, and flk-2/flt-3 ligand taken from CD34+ hematopoietic progenitors that were mobilized into the peripheral blood of cancer patients treated with high-dose cyclophosphamide and filgrastim. The target cells used for gene delivery were either CD34+ cells that had been subsequently induced to differentiate into mature DCs or DCs transduced after ex vivo generation from CD34+ cells. The results showed that: (a) infection of CD34+ cell derived-DCs (mature DCs) with either viral vector resulted in the efficient synthesis of **recombinant** protein, and (b) CD34+ cells were permissive for the expression of the **recombinant** reporter gene after infection with Ad-MFG-AP but not after infection with **MVA**-P11 LZ. In conclusion, these results suggest that vaccinia and adenovirus vectors are candidate to act as vehicles in genetically engineering human DCs.

L2 ANSWER 33 OF 63 MEDLINE DUPLICATE 22
AN 1998131961 MEDLINE
DN 98131961
TI Highly attenuated modified vaccinia **virus Ankara** replicates in baby hamster kidney cells, a potential host for **virus** propagation, but not in various human transformed and primary cells.
AU Drexler I; Heller K; Wahren B; Erfle V; Sutter G
CS Institut fur Molekulare Virologie, GSF-Forschungszentrum fur Umwelt und Gesundheit GmbH, Munchen-Neuherberg, Germany.
SO JOURNAL OF GENERAL VIROLOGY, (1998 Feb) 79 (Pt 2) 347-52.
Journal code: I9B. ISSN: 0022-1317.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199804
EW 19980404
AB Although desirable for safety reasons, the host range restrictions of modified vaccinia **virus Ankara** (**MVA**) make it less applicable for general use. Propagation in primary chicken embryo fibroblasts (CEF) requires particular cell culture experience and has no pre-established record of tissue culture reproducibility. We investigated a variety of established cell lines for productive **virus** growth

and **recombinant** gene expression. Baby hamster kidney cells (BHK), a well-characterized, easily maintained cell line, supported **MVA** growth and as proficient expression of the E. coli lacZ reporter gene as the highly efficient CEF, whereas other cell lines were non-permissive or allowed only very limited **MVA** replication. Importantly, no **virus** production occurred in patient-derived infected primary human cells. These results emphasize the safety and now improved accessibility of **MVA** for the development of expression vectors and live **recombinant** vaccines.

L2 ANSWER 34 OF 63 MEDLINE DUPLICATE 23
AN 1999058184 MEDLINE
DN 99058184
TI Marker rescue of the host range restriction defects of modified vaccinia **virus Ankara**.
AU Wyatt L S; Carroll M W; Czerny C P; Merchlinsky M; Sisler J R; Moss B
CS National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, 20892, USA.
SO VIROLOGY, (1998 Nov 25) 251 (2) 334-42.
Journal code: XEA. ISSN: 0042-6822.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199903
EW 19990301
AB The severely attenuated and host range (HR) restricted modified vaccinia **virus Ankara** (**MVA**) was derived by >500 passages in chick embryo fibroblasts, during which multiple deletions and mutations occurred. To determine the basis of the HR defect, we prepared cosmids from the parental vaccinia **virus Ankara** genome and transfected them into nonpermissive monkey BS-C-1 cells that had been infected with **MVA**. **Recombinant** viruses that formed macroscopic plaques were detected after transfections with DNA segments that mapped near the left end of the viral genome. Plaque-forming viruses, generated by transfections with four individual cosmids and one pair of minimally overlapping cosmids, were purified, and their HRs were evaluated in BS-C-1 cells, rabbit RK-13 cells, and human HeLa, MRC-5, and A549 cells. The acquisition of the K1L and SPI-1 HR genes and the repair of large deletions were determined by polymerase chain reaction or pulse-field gel electrophoresis of NotI restriction enzyme digests of genomic DNA. The following results indicated the presence of previously unrecognized vaccinia **virus** HR genes: (1) the major mutations that restrict HR are within the left end of the **MVA** genome because the phenotypes of some recombinants approached that of the parental **virus**, (2) acquisition of the K1L gene correlated with the ability of **recombinant** viruses to propagate in RK-13 cells but did not enhance replication in human or monkey cell lines, (3) acquisition of the SPI-1 gene correlated with **virus** propagation in A549 cells but not with the extent of **virus** spread in monkey or other human cell lines, (4) there are at least two impaired HR genes because rescue occurred with nonoverlapping or minimally overlapping cosmids and **recombinant** viruses with intermediate HRs were isolated, and (5) at least one of the new HR genes did not map within any of the major deletions because the size of the left terminal NotI fragment was not appreciably altered in some **recombinant** viruses.

L2 ANSWER 35 OF 63 MEDLINE DUPLICATE 24
AN 1998229452 MEDLINE
DN 98229452
TI Mechanisms of replication-deficient vaccinia **virus**/T7 RNA polymerase hybrid expression: effect of T7 RNA polymerase levels and alpha-amanitin.
AU Engleka K A; Lewis E W; Howard B H
CS Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda,

Maryland 20892-2753, USA.. engleka1@jeflin.tju.edu
SO VIROLOGY, (1998 Apr 10) 243 (2) 331-9.
Journal code: XEA. ISSN: 0042-6822.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199807
EW 19980705
AB Components of the eukaryotic vaccinia **virus**/T7 RNA polymerase hybrid expression system were assessed using **recombinant** and nonrecombinant forms of modified vaccinia **Ankara** (**MVA**), a replication-deficient vaccinia **virus** strain. **Recombinant MVA virus** expressing T7 RNA polymerase (Wyatt, L. S., Moss, B., and Rozenblatt, S. (1995). Virology 210, 202-205) stimulated high levels of expression from a T7 promoter-chloramphenicol acetyltransferase (CAT) reporter. Most, but not all, of the virally induced expression was T7 RNA polymerase and T7 promoter dependent, with no viral enhancement of translation of T7 transcripts. The efficacy of supplying T7 RNA polymerase expression from nonviral sources was evaluated using a self-amplifying T7 RNA polymerase autogene or an inducible T7 RNA polymerase expression vector. The latter modes yielded CAT activity dependent on T7 RNA polymerase expression; however, expression required viral factors independent of T7 RNA polymerase and did not reach that attained using the **recombinant virus**. In further experiments, **MVA**-induced T7 RNA polymerase expression was upregulated by alpha-amanitin, an inhibitor of eukaryotic polymerases. This indicates that **MVA**/T7 RNA polymerase hybrid expression may be rendered still more efficient by ameliorating transcriptional interference due to an alpha-amanitin-sensitive eukaryotic factor(s).

L2 ANSWER 36 OF 63 MEDLINE DUPLICATE 25
AN 1998391505 MEDLINE
DN 98391505
TI Characterization of a **recombinant** human calicivirus capsid protein expressed in mammalian cells.
AU Pletneva M A; Sosnovtsev S V; Sosnovtseva S A; Green K Y
CS Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
SO VIRUS RESEARCH, (1998 Jun) 55 (2) 129-41.
Journal code: X98. ISSN: 0168-1702.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199901
EW 19990104
AB The capsid protein of the Hawaii strain of human calicivirus was expressed in the transient **MVA**/bacteriophage T7 polymerase hybrid expression system in order to examine its processing in mammalian cells. Selected amino acid modifications (an insertion, deletion, and substitution) at the predicted amino terminus of the capsid protein as well as the presence or absence of the ORF3 gene were examined for their effect on capsid expression. The protein was expressed efficiently in cell lines derived from three different species, with most of the expressed protein remaining localized within the cells. There was no evidence for N-linked glycosylation or myristylation of the 57 kDa capsid protein. Hawaii **virus**-like particles (HV VLPs), efficiently produced in the baculovirus expression system, were not observed in this expression system under the conditions in this study.

L2 ANSWER 37 OF 63 MEDLINE DUPLICATE 26
AN 1998120825 MEDLINE
DN 98120825
TI Immunogenicities of intravenous and intramuscular administrations of

modified vaccinia **virus Ankara**-based multi-CTL epitope vaccine for human immunodeficiency **virus** type 1 in mice.

AU Hanke T; Blanchard T J; Schneider J; Ogg G S; Tan R; Becker M; Gilbert S C; Hill A V; Smith G L; McMichael A

CS Molecular Immunology Group, Institute of Molecular Medicine, University of Oxford, The John Radcliffe, UK.. hanke@ermine.ox.ac.uk

SO JOURNAL OF GENERAL VIROLOGY, (1998 Jan) 79 (Pt 1) 83-90. Journal code: I9B. ISSN: 0022-1317.

CY ENGLAND: United Kingdom

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals; Cancer Journals

EM 199804

EW 19980404

AB A vaccine against human immunodeficiency **virus** (HIV) is still awaited. Although the correlates of protection remain elusive, it is likely that CD8+ T cells play an important role in the control of this infection. To firmly establish the importance of these cells in protective immunity, a means of efficient elicitation of CD8+ T cell responses in the absence of antibody is needed and, when available, might represent a crucial step towards a protective vaccine. Here, a novel vaccine candidate was constructed as a multi-cytotoxic T lymphocyte (CTL) epitope gene delivered and expressed using modified vaccinia **virus Ankara** (**MVA**). The immunogen consists of 20 human, one murine and three rhesus macaque epitopes. The non-human epitopes were included so that the vaccine can be tested for immunogenicity and optimal vaccination doses, routes and regimes in experimental animals. Mice were immunized intravenously (i.v.) or intramuscularly (i.m.) using a single dose of 10(6) p.f.u. of the **recombinant MVA** and the induction of CTL was assessed. It was demonstrated that both administration routes induced specific CTL responses and that the i.v. route was moderately more immunogenic than the i.m. route. The frequencies of ex vivo splenocytes producing interferon-y upon MHC class I-restricted peptide stimulation were determined using an ELISPOT assay. Also, the correct processing and presentation of some HLA-restricted epitopes in human cells was confirmed.

L2 ANSWER 38 OF 63 MEDLINE DUPLICATE 27

AN 1999036460 MEDLINE

DN 99036460

TI Rapid and efficient recovery of Sendai **virus** from cDNA: factors influencing **recombinant virus** rescue.

AU Leyrer S; Neubert W J; Sedlmeier R

CS Max-Planck-Institut fur Biochemie, Abteilung Virusforschung, Martinsried, Germany.

SO JOURNAL OF VIROLOGICAL METHODS, (1998 Nov) 75 (1) 47-58. Journal code: HQR. ISSN: 0166-0934.

CY Netherlands

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199903

EW 19990305

AB In a comparative study the factors influencing the recovery of **recombinant** Sendai viruses (SeV) from plasmid based cDNA were analysed systematically in order to establish an efficient and robust method for **virus** rescue. The amounts and ratios of transfected helper plasmids encoding the viral N, P and L proteins proved to be crucial for **virus** rescue, and they were optimised step-by-step for enhanced **virus** release. When the C open reading frame from the P gene was expressed at low level, **virus** rescue was generally possible but **virus** release could be improved when C gene expression was abolished completely. SeV particle formation could be increased greatly when the transcription initiation site for T7 polymerase in the cDNA was modified or when the genomic ribozyme instead of the antigenomic ribozyme of hepatitis delta **virus** was used for

processing the 3'end of the viral RNA transcript. Heterologous helper viruses vTF7-3 and **MVA**-T7, which are necessary for T7 polymerase production in transfected cells, were compared for their use in SeV recovery and subsequent elimination of the helper **virus** from **recombinant** SeV. Interference with SeV replication was less severe with **MVA**-T7, and **MVA**-T7 was eliminated efficiently without the need for any inhibitors by serial passages in Vero cells. Optimal combination of all parameters led to a highly efficient generation of **recombinant** SeV from cDNA. Titres of the released **virus** particles are high enough to enable analysis of the **recombinant** SeV directly on test cells or propagation in cell cultures without the need for amplification in embryonated chicken eggs. The system is very robust and allows rapid generation of defined SeV mutants that require specialised host cells for propagation.

L2 ANSWER 39 OF 63 AIDSLINE
AN 1997:21310 AIDSLINE
DN AIDS-97927073
TI Vaccinia **virus** strain Modified **Virus Ankara**: characterisation of cytokine receptor profile, virological features, and use as an immunological reagent.
AU Blanchard T; Alcami A; Becker M; Hanke T; Andrea P; Gould K; Britton W; Anderton P; Rowland-Jones S; McMichael A J; Smith G L
CS Sir William Dunn School of Pathology, Oxford, England. Fax: +44 1865 275501.
SO Conf Adv AIDS Vaccine Dev, (1997). pp. 108 (Poster 3).
CY United States
DT (MEETING ABSTRACTS)
FS AIDS
LA English
EM 199711
AB Modified **Virus Ankara** (**MVA**) is an attenuated strain of vaccinia **virus** derived by serial passage through chick cells. **MVA** was administered to greater than 120,000 individuals towards the end of the smallpox eradication campaign without any significant adverse effects. We have sought to establish the reasons for the safety and immunogenicity of this human vaccine candidate, and have generated recombinants for use in immunological research. Modified **Virus Ankara** has no functional interferon alpha/beta receptor, interferon gamma receptor or TNF receptor, but it does possess a functional IL-1beta receptor. **MVA** is the only vaccinia known to possess this favourable cytokine receptor profile. **MVA** is able to form extracellular enveloped viral particles. It is also able to replicate to a limited extent in human cells. We have found that **recombinant MVA** can be made by disruption of the TK locus using the standard pSC11 vector. This has enabled us to generate a wide range of recombinants expressing proteins from influenza, SIV, HIV, and other pathogens; in addition we have generated polyepitope constructs. Studies with influenza NP have shown epitope-specific block in class I presentation in the same fashion as seen with other strains of vaccinia. The NP **MVA recombinant** has been successfully used to restimulate human peripheral blood cytotoxic T cells. A polyepitope construct generated at least 5 HIV-1 clade B class I epitopes. Immunisation studies in mice show that the iv route elicits stronger CTL responses than the im route; primate work is in progress. **Recombinant MVA** is a promising human vaccine candidate.

L2 ANSWER 40 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1997:168617 CAPLUS
DN 126:153677
TI **Recombinant** vaccinia **virus** for expression of foreign genes for therapeutical applications
IN Sutter, Gerd; Ohlmann, Marion; Erfle, Volker
PA GSF-Forschungszentrum fuer Umwelt und Gesundheit GmbH, Germany; Sutter, Gerd; Ohlmann, Marion; Erfle, Volker
SO PCT Int. Appl., 45 pp.

CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9702355	A1	19970123	WO 1996-EP2926	19960703
	W: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	CA 2225278	AA	19970123	CA 1996-2225278	19960703
	AU 9666110	A1	19970205	AU 1996-66110	19960703
	EP 836648	A1	19980422	EP 1996-925654	19960703
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI				
	CN 1189857	A	19980805	CN 1996-195254	19960703
	BR 9609303	A	19990525	BR 1996-9303	19960703
	JP 11509091	T2	19990817	JP 1996-504824	19960703
	NO 9800026	A	19980102	NO 1998-26	19980102
PRAI	DK 1995-782	19950704			
	WO 1996-EP2926	19960703			

AB Disclosed are **recombinant** vaccinia viruses derived from the modified vaccinia viruses **Ankara** (**MVA**), which contain and express foreign genes that are inserted at the site of a naturally occurring deletion in the **MVA** genome, and use of the **recombinant MVA** viruses for the prodn. of polypeptides, e.g. antigens or therapeutic agents. Also disclosed is the prodn. of **recombinant** viruses for vaccines, or viral vectors for gene therapy. Prepn. of **recombinant virus MVA** -LAInef expressing the nef gene of HIV-1 strain LAI was shown.

L2 ANSWER 41 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1997:155039 CAPLUS
DN 126:153660
TI Double marker selection methods for the construction of wild-type-free viral vectors for use in vaccines
IN Scheiflinger, Friedrich; Antoine, Gerhard; Falkner, Falko-Guenter; Dorner, Friedrich; Eibl, Johan
PA Immuno Ag, Austria
SO Eur. Pat. Appl., 68 pp.
CODEN: EPXXDW
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	EP 753581	A1	19970115	EP 1995-110727	19950710
	R: AT, BE, CH, DE, DK, ES, FR, GB, IE, IT, LI, NL, SE				

AB A method (transient marker stabilization) of inserting a gene into a viral vector using a combination of a selectable marker and a color marker to minimize the recovery of false positives and wild-type **virus** is described. The insertion cassette contains the foreign DNA and a dominant selection marker gene and a color selection marker gene and a pair of direct repeat sequences. Some or all of the foreign DNA segment is not flanked by the direct repeats. This is introduced into the vector backbone and a preliminary selection and screening for both markers is made. Selected **virus** are then propagated without selection and then screened for the loss of both markers. Viruses lacking both markers are then screened for the presence of the foreign DNA insert. The foreign may include genes for antigens and immunomodulators such as interleukin 6 or interferon .gamma.. Furthermore, the invention relates to DNA mols. for said method as well as **recombinant** viruses produced by said method and vaccines comprising said viruses.

L2 ANSWER 42 OF 63 MEDLINE DUPLICATE 28
AN 97151114 MEDLINE
DN 97151114
TI Rescue of rinderpest **virus** from cloned cDNA.
AU Baron M D; Barrett T
CS Institute for Animal Health, Pirbright, Surrey, United Kingdom.. michael.baron@bbsrc.ac.uk
SO JOURNAL OF VIROLOGY, (1997 Feb) 71 (2) 1265-71.
Journal code: KCV. ISSN: 0022-538X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199704
EW 19970404
AB Rinderpest **virus** is a morbillivirus and is the causative agent of a widespread and important disease of cattle. The viral genome is a single strand of RNA in the negative sense. We have constructed plasmids containing cDNA copies of the 5' and 3' termini of the **virus** separated by a reporter gene and have shown that antigenome-sense RNA transcripts of these model genomes can be replicated, transcribed, and packaged by helper **virus**, both rinderpest **virus** and the related measles **virus**. Further, these genome analogs can be replicated and transcribed by viral proteins expressed from cDNA clones by using a **recombinant** vaccinia **virus** expressing T7 RNA polymerase (**MVA**-T7). Using this latter system, we have rescued live rinderpest **virus** from a full-length cDNA copy of the genome of the RBOK vaccine strain. The **recombinant virus** appears to grow in tissue culture identically to the original **virus**.

L2 ANSWER 43 OF 63 MEDLINE DUPLICATE 29
AN 97286006 MEDLINE
DN 97286006
TI Highly attenuated modified vaccinia **virus Ankara** (**MVA**) as an effective **recombinant** vector: a murine tumor model.
AU Carroll M W; Overwijk W W; Chamberlain R S; Rosenberg S A; Moss B; Restifo N P
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
SO VACCINE, (1997 Mar) 15 (4) 387-94.
Journal code: X6O. ISSN: 0264-410X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199709
EW 19970902
AB Modified vaccinia **virus Ankara** (**MVA**), a highly attenuated strain of vaccinia **virus** (VV) that is unable to replicate in most mammalian cells, was evaluated as an expression vector for a model tumor associated antigen (TAA) and as a potential anti-cancer vaccine. We employed an experimental murine model in which an adenocarcinoma tumor line, CT26.CL25, was stably transfected with a model TAA, beta-galactosidase (beta-gal). Mice injected intramuscularly with a **recombinant MVA** (rMVA) expressing beta-gal (**MVA** -LZ), were protected from a lethal intravenous (i.v.) challenge with CT26.CL25. In addition, splenocytes from mice primed with **MVA**-LZ were therapeutically effective upon adoptive transfer to mice bearing pulmonary metastases of the CT26.CL25 tumor established 3 days earlier. Most importantly, i.v. inoculation with **MVA**-LZ resulted in significantly prolonged survival of mice bearing three day old pulmonary metastases. This prolonged survival compared favorably to mice treated with a replication competent **recombinant** VV expressing beta-gal.

These findings indicate that rMVA is an efficacious alternative to the more commonly used replication competent VV for the development of new **recombinant** anti-cancer vaccines.

L2 ANSWER 44 OF 63 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.
AN 97302311 EMBASE
DN 1997302311
TI Enhanced immunogenicity for CTL induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia **virus Ankara** encoding pre-erythrocytic.
AU Schneider J.; Gilbert S.C.; Blanchard T.; Hanke T.; Sinden R.; Robson K.J.; Smith G.; Hill A.V.S.
CS J. Schneider, Institute of Molecular Medicine, Nuffield Department of Medicine, John Radcliffe Hospital, Oxford, United Kingdom
SO Immunobiology, (1997) 197/2-4 (352-353).
Refs: 0
ISSN: 0171-2985 CODEN: ZIMMDO
CY Germany
DT Journal; Conference Article
FS 004 Microbiology
026 Immunology, Serology and Transplantation
037 Drug Literature Index
039 Pharmacy
LA English

L2 ANSWER 45 OF 63 MEDLINE DUPLICATE 30
AN 1998063240 MEDLINE
DN 98063240
TI Host range and cytopathogenicity of the highly attenuated **MVA** strain of vaccinia **virus**: propagation and generation of **recombinant** viruses in a nonhuman mammalian cell line.
AU Carroll M W; Moss B
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0445, USA.
SO VIROLOGY, (1997 Nov 24) 238 (2) 198-211.
Journal code: XEA. ISSN: 0042-6822.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199803
EW 19980303
AB Modified vaccinia **virus Ankara** (**MVA**), attenuated by over 500 passages in primary chick embryo fibroblasts (CEF), is presently being used as a safe expression vector. We compared the host ranges of **MVA** and the parental **Ankara** strain in CEF and 15 permanent cell lines. The cells could be grouped into three categories: permissive, semipermissive, and nonpermissive. For **MVA**, the permissive category consisted of primary CEF, a quail cell line derived from QT6, and the Syrian hamster cell line BHK-21. Only in BHK-21 cells did the **virus** yield approach that occurring in primary CEF. The semipermissive category included two African green monkey cell lines: BS-C-1 and CV-1. The nonpermissive category for **MVA** consisted of three human cell lines HeLa, 293, and SW 839; one rhesus monkey cell line FRhK-4; two Chinese hamster cell lines CHO and CHL; one pig cell line PK(15); and three rabbit cell lines RK13, RAB-9, and SIRC. The grouping for **MVA** with a restored K1L host range gene was similar except for the inclusion of RK13 cells among permissive lines. The grouping for the **Ankara** strain, however, was quite different with more permissive and semipermissive cell lines. Nevertheless, in cells that were permissive for **MVA**, the **virus** replicated to higher levels than **Ankara**, consistent with both positive and negative growth elements associated with the adaptation of **MVA**. The cell lines were also characterized according to their susceptibility to **MVA**-induced cytopathic effects, expression of a late promoter

regulated reporter gene by an **MVA recombinant**, and stage at which virion morphogenesis was blocked. Finally, the permissive BHK-21 cell line was shown to be competent for constructing and propagating **recombinant MVA**, providing an alternative to primary CEF.

L2 ANSWER 46 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1997:17328 CAPLUS
DN 126:113790
TI Rescue of measles **virus** using a replication-deficient vaccinia-T7 vector
AU Schneider, Henriette; Spielhofer, Pius; Kaelin, Karin; Doetsch, Christina; Radecke, Frank; Sutter, Gerd; Billeter, Martin A.
CS Institut fuer Molekularbiologie I, Universitaet Zuerich, Hoenggerberg, Zurich, 8093, Switz.
SO J. Virol. Methods (1997), 64(1), 57-64
CODEN: JVMEDH; ISSN: 0166-0934
PB Elsevier
DT Journal
LA English
AB A system which allows the reconstitution of measles **virus** (MV) from cloned cDNA is described. The severely host cell restricted vaccinia vector **MVA**-T7 expressing bacteriophage T7 RNA polymerase was used to generate full-length antigenomic MV RNA and simultaneously the mRNAs encoding the viral N, P and L proteins in order to produce replicationally and transcriptionally active nucleocapsids. The functionality of the N, P and L proteins was demonstrated first by their ability to rescue MV specific subgenomic RNAs. Assembly and budding of reconstituted MV was shown by syncytia formation and subsequently by **virus** isolation. The inability of **MVA**-T7 to produce progeny **virus** in most mammalian cells circumvents the necessity to sep. the reconstituted MV from the **MVA**-T7 helper **virus**. Since all components are expressed transiently, this system is esp. suitable for studying the functions of N, P and L. Furthermore, it is useful for investigating later steps in the MV life cycle.

L2 ANSWER 47 OF 63 AIDSLINE
AN 1996:11025 AIDSLINE
DN AIDS-96920213
TI SIV replication dynamics as a major determinant of disease outcome in infected macaques: vaccine modulation.
AU Hirsch V M; Fuerst T R; Sutter G; Yang L C; Goldstein S; Piatak M Jr; Elkins W R; Moss B; Montefiori D C; Lifson J D
CS NIAID, NIH, Rockville, MD.
SO 3rd Conf Retro and Opportun Infect, (1996). pp. 92.
CY United States
DT (MEETING ABSTRACTS)
FS AIDS
LA English
EM 199611
AB Viral replication dynamics were explored in a group of SIV-infected macaques that exhibited different disease courses ranging from rapid AIDS to apparent non-progression. Sequential plasma, PBMC and lymph node samples from a group of twelve SIVsmE660 infected rhesus macaques that had received prior immunization with non-**recombinant** or trivalent (gag-pol, env) SIV **recombinant** vaccinia viruses were analyzed by quantitative competitive PCR (QC-PCR) and other viral load assays. Longitudinal viral load data were evaluated along with lymphocyte subsets, neutralizing antibody responses, sequential NV-specific in situ hybridization of lymph node biopsies and clinical outcome. Viral replication during and following primary viremia showed 3 patterns, correlated with clinical outcome. High primary (greater thanl0 (6) copy equiv./ml) and subsequent increasing plasma viremia (up to 2 x 10(8)/ml) correlated with rapid progression to AIDS (n=2). Most animals exhibited substantial primary viremia (10(5)to 10(6)/copy equiv./ml) a transient one log decline, and subsequent progressive increase in viremia in the

post-acute phase of infection with progression to AIDS within a year (n=6). Low levels of primary plasma viremia (10(4) to 10(5) copy equiv./ml) followed by sustained limitation of viral replication (less than 10(4) copy equiv./ml), was associated with maintenance of normal lymphocyte subsets and intact lymphoid architecture (n=4), as seen in HIV-1 infected long-term nonprogressors. Three of the macaques that showed this last pattern had been immunized with a SIV **recombinant** of the attenuated vaccinia **virus**, modified vaccinia **Ankara** (**MVA**-SIV). The dynamics of **virus** replication are closely linked to disease course; sustained suppression of **virus** appears to promote long-term, asymptomatic survival of SIV-infected macaques.

L2 ANSWER 48 OF 63 SCISEARCH COPYRIGHT 2000 ISI (R)
AN 96:390134 SCISEARCH
GA The Genuine Article (R) Number: UL104
TI EXTRACELLULAR VACCINIA **VIRUS** ENVELOPE GLYCOPROTEIN ENCODED BY THE A33R GENE
AU ROPER R L; PAYNE L G; MOSS B (Reprint)
CS NIAID, VIRAL DIS LAB, NIH, BLDG 4, ROOM 229, 9000 ROCKVILLE PIKE, BETHESDA, MD, 20892 (Reprint); NIAID, VIRAL DIS LAB, NIH, BETHESDA, MD, 20892; INST VIRUS RES, CAMBRIDGE, MA, 02138
CYA USA
SO JOURNAL OF VIROLOGY, (JUN 1996) Vol. 70, No. 6, pp. 3753-3762. ISSN: 0022-538X.
DT Article; Journal
FS LIFE
LA ENGLISH
REC Reference Count: 46
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB With the aid of three monoclonal antibodies (MAbs), a glycoprotein specifically localized to the outer envelope of vaccinia **virus** was shown to be encoded by the A33R gene. These MAbs reacted with a glycosylated protein that migrated as 23- to 28-kDa and 55-kDa species under reducing and nonreducing conditions, respectively. The protein recognized by the three MAbs was synthesized by all 11 orthopoxviruses tested: eight strains of vaccinia **virus** (including modified vaccinia **virus Ankara**) and one strain each of cowpox, rabbitpox, and ectromelia viruses. The observation that the protein synthesized by ectromelia **virus**-infected cells reacted with only one of the three MAbs provided a means of mapping the gene encoding the glycoprotein, By transfecting vaccinia **virus** DNA into cells infected with ectromelia **virus** and assaying for MAb reactivity, we mapped the glycoprotein to the A33R open reading frame. The amino acid sequence and hydrophilicity plot predicted that the A33R gene product is a type II membrane protein with two asparagine-linked glycosylation sites. Triton X-114 partitioning experiments indicated that the A33R gene product is an integral membrane protein. The ectromelia **virus** homolog of the vaccinia **virus** A33R gene was sequenced, revealing 90% predicted amino acid identity. The vaccinia and variola **virus** homolog sequences predict 94% identical amino acids, the latter having one fewer internal amino acid. Electron microscopy revealed that the A33R gene product is expressed on the surface of extracellular enveloped virions but not on the intracellular mature form of **virus**. The conservation of this protein and its specific incorporation into viral envelopes suggest that it is important for **virus** dissemination.

L2 ANSWER 49 OF 63 MEDLINE DUPLICATE 31
AN 96211507 MEDLINE
DN 96211507
TI Patterns of viral replication correlate with outcome in simian immunodeficiency **virus** (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia **virus Ankara**.
AU Hirsch V M; Fuerst T R; Sutter G; Carroll M W; Yang L C; Goldstein S; Piatak M Jr; Elkins W R; Alvord W G; Montefiori D C; Moss B; Lifson J D

CS Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20892, USA.
NC AI35467 (NIAID)
SO JOURNAL OF VIROLOGY, (1996 Jun) 70 (6) 3741-52.
Journal code: KCV. ISSN: 0022-538X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199609
AB The dynamics of plasma viremia were explored in a group of 12 simian immunodeficiency **virus** (SIV)-infected rhesus macaques (Macaca mulatta) that had received prior immunization with either nonrecombinant or trivalent (gag-pol, env) SIV-**recombinant** vaccinia viruses. Three distinct patterns of viral replication observed during and following primary viremia accounted for significant differences in survival times. High-level primary plasma viremia with subsequently increasing viremia was associated with rapid progression to AIDS (n = 2). A high-level primary plasma **virus** load with a transient decline and subsequent progressive increase in viremia in the post-acute phase of infection was associated with progression to AIDS within a year (n = 6). Low levels of primary plasma viremia followed by sustained restriction of **virus** replication were associated with maintenance of normal lymphocyte subsets and intact lymphoid architecture (n = 4), reminiscent of the profile observed in human immunodeficiency **virus** type 1-infected long-term nonprogressors. Three of four macaques that showed this pattern had been immunized with an SIV **recombinant** derived from the attenuated vaccinia **virus**, modified vaccinia **virus Ankara**. These data link the dynamics and extent of **virus** replication to disease course and suggest that sustained suppression of **virus** promotes long-term, asymptomatic survival of SIV-infected macaques. These findings also suggest that vaccine modulation of host immunity may have profound beneficial effects on the subsequent disease course, even if sterilizing immunity is not achieved.

L2 ANSWER 50 OF 63 MEDLINE DUPLICATE 32
AN 97147444 MEDLINE
DN 97147444
TI Development of a replication-deficient **recombinant** vaccinia **virus** vaccine effective against parainfluenza **virus** 3 infection in an animal model.
AU Wyatt L S; Shors S T; Murphy B R; Moss B
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0445, USA.
SO VACCINE, (1996 Oct) 14 (15) 1451-8.
Journal code: X6O. ISSN: 0264-410X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199706
EW 19970603
AB The highly attenuated, replication-deficient, modified vaccinia **virus Ankara** (**MVA**) was used to express the fusion (F) and/or hemagglutinin-neuraminidase (HN) glycoproteins of parainfluenza **virus** 3 (PIV3). Initial **recombinant** viruses in which the HN gene was regulated by a very strong synthetic earlyllate promoter replicated poorly in permissive chick embryo cells evidently due to toxic levels of the gene product. This result led us to construct and evaluate a modified earlyllate promoter derived from the H5 gene of vaccinia **virus**. Reporter gene experiments indicated that the enhanced H5 promoter was about five times stronger than the 7.5 promoter used in previous **recombinant** vaccinia/ PIV3 viruses. Although the overall expression from the modified H5 promoter was less than that of the strong synthetic promoter, early expression, determined in the presence of an inhibitor of DNA replication, was higher.

Importantly, **recombinant MVA** employing the modified H5 promoter to regulate the F or HN gene of PIV3 replicated to high titers in chick cells and expressed functional F or HN proteins as measured by syncytial formation upon dual infection of mammalian cells. Cotton rats inoculated with **recombinant MVA** expressing F or HN by intramuscular or intranasal routes produced high levels of antibody. The **virus** expressing HN, however, was the more effective of the two in inducing immunity to PIV3 challenge, reducing PIV3 viral titers in the nasal turbinates by at least 4.7 logs and in the lungs by 3.4 logs, similar to that achieved by immunization with PIV3. These studies support further testing of **recombinant MVA**/PIV3 viruses as safe and effective candidate vaccines.

L2 ANSWER 51 OF 63 MEDLINE DUPLICATE 33
AN 96201441 MEDLINE
DN 96201441
TI Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a **recombinant** fowlpox **virus**.
AU Britton P; Green P; Kottier S; Mawditt K L; Penzes Z; Cavanagh D; Skinner M A
CS Division of Molecular Biology, Institute for Animal Health, Compton, Newbury, Berkshire, UK.
SO JOURNAL OF GENERAL VIROLOGY, (1996 May) 77 (Pt 5) 963-7.
Journal code: I9B. ISSN: 0022-1317.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199608
AB The bacteriophage T7 RNA polymerase gene was integrated into the fowlpox **virus** genome under the control of the vaccinia **virus** early/late promoter, P7.5. The **recombinant** fowlpox **virus** , fpEFLT7pol, stably expressed T7 RNA polymerase in avian and mammalian cells, allowing transient expression of transfected genes under the control of the T7 promoter. The **recombinant** fowlpox **virus** expressing T7 RNA polymerase offers an alternative to the widely used vaccinia **virus** vTF7-3, or the recently developed modified vaccinia **virus Ankara** (**MVA**) T7 RNA polymerase **recombinant**, a highly attenuated strain with restricted host-range. **Recombinant** fowlpox viruses have the advantage that as no infectious **virus** are produced from mammalian cells they do not have to be used under stringent microbiological safety conditions.

L2 ANSWER 52 OF 63 MEDLINE DUPLICATE 34
AN 97080498 MEDLINE
DN 97080498
TI Characterization of the vaccinia **MVA** hemagglutinin gene locus and its evaluation as an insertion site for foreign genes.
AU Antoine G; Scheiflinger F; Holzer G; Langmann T; Falkner F G; Dorner F
CS IMMUNO AG, Biomedical Research Center, Orth/Donau, Austria.
SO GENE, (1996 Oct 24) 177 (1-2) 43-6.
Journal code: FOP. ISSN: 0378-1119.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
OS GENBANK-X91135
EM 199702
EW 19970204
AB The 'Modified Vaccinia **Ankara**' (**MVA**) strain is a potential live vaccine vector. The use of the hemagglutinin (ha) gene of the **MVA** strain as an insertion site for foreign genes was evaluated. To identify the molecular basis of the hemagglutinin-negative (HA-) phenotype of **MVA**, the ha gene and the region around this gene were sequenced. Amino acid (aa) sequence comparisons with functional

hemagglutinins of other vaccinia strains predicted a functional polypeptide. The late part of the promoter region of the ha gene, however, was deleted, causing the apparent loss of the ha gene function. Nevertheless, insertion of foreign DNA into the ha gene allowed generation of functional **recombinant** viruses, indicating that the ha-gene region is a suitable insertion site.

L2 ANSWER 53 OF 63 MEDLINE DUPLICATE 35
AN 96342131 MEDLINE
DN 96342131
TI Host range restricted, non-replicating vaccinia **virus** vectors as vaccine candidates.
AU Moss B; Carroll M W; Wyatt L S; Bennink J R; Hirsch V M; Goldstein S; Elkins W R; Fuerst T R; Lifson J D; Piatak M; Restifo N P; Overwijk W; Chamberlain R; Rosenberg S A; Sutter G
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.
SO ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, (1996) 397 7-13. Ref: 35
Journal code: 2LU. ISSN: 0065-2598.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Priority Journals
EM 199703
EW 19970304
AB Three model systems were used to demonstrate the immunogenicity of highly attenuated and replication-defective **recombinant MVA**.
(1) Intramuscular inoculation of **MVA**-IN-Fha/np induced humoral and cell-mediated immune responses in mice and protectively immunized them against a lethal respiratory challenge with influenza **virus**.
Intranasal vaccination was also protective, although higher doses were needed. (2) In rhesus macaques, an immunization scheme involving intramuscular injections of **MVA**-SIVenv/gag/pol greatly reduced the severity of disease caused by an SIV challenge. (3) In a murine cancer model, immunization with **MVA**-beta gal prevented the establishment of tumor metastases and even prolonged life in animals with established tumors. These results, together with previous data on the safety of **MVA** in humans, suggest the potential usefulness of **recombinant MVA** for prophylactic vaccination and therapeutic treatment of infectious diseases and cancer.

L2 ANSWER 54 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1995:549387 CAPLUS
DN 122:288910
TI Multipotent paramunity inducers based on combinations of pox **virus** components, process for their production and their therapeutic use
IN Mayr, Anton
PA Germany
SO Ger., 18 pp.
CODEN: GWXXAW
DT Patent
LA German
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	DE 4405841	C1	19950105	DE 1994-4405841	19940223
	EP 669133	A1	19950830	EP 1994-103047	19940301
	EP 669133	B1	19970521		
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE				
	AT 153242	E	19970615	AT 1994-103047	19940301
	ES 2102081	T3	19970716	ES 1994-103047	19940301
	WO 9522978	A1	19950831	WO 1995-EP160	19950117
	W: AU, BR, CA, CN, FI, HU, JP, KR, NO, NZ, PL, RU, US				
	CA 2182207	AA	19950831	CA 1995-2182207	19950117
	AU 9514167	A1	19950911	AU 1995-14167	19950117

	AU 690625	B2	19980430		
	CN 1142187	A	19970205	CN 1995-191784	19950117
	JP 09504803	T2	19970513	JP 1995-522088	19950117
	JP 2873880	B2	19990324		
	HU 75545	A2	19970528	HU 1996-1947	19950117
	BR 9506882	A	19970819	BR 1995-6882	19950117
	NO 9603462	A	19960820	NO 1996-3462	19960820
	FI 9603277	A	19960822	FI 1996-3277	19960822
PRAI	DE 1994-4405841	19940223			
	WO 1995-EP160	19950117			

AB Multipotent paramunity (non-antigen-specific) inducers are described which are based on combinations of **recombinant** viral polypeptides from 2 or more different pox **virus** strains with paramunizing properties. These multipotent paramunity inducers have almost no immunogenic activity, but they do have very strong paramunizing activity, so that repeated and continuous applications are possible. Thus, paramunity inducers were produced using the HP1 fowlpox **virus** strain attenuated by passage in chick embryo fibroblasts and inactivated with .beta.-propiolactone.

L2 ANSWER 55 OF 63 MEDLINE DUPLICATE 36
AN 96121836 MEDLINE
DN 96121836
TI Interferon induction in peripheral blood mononuclear leukocytes of man and farm animals by poxvirus vector candidates and some poxvirus constructs.
AU Buttner M; Czerny C P; Lehner K H; Wertz K
CS Institut fur Medizinische Microbiologie, Infections und Seuchenmedizin, Munich, Germany.
SO VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, (1995 Jun) 46 (3-4) 237-50. Journal code: XCB. ISSN: 0165-2427.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199603
AB Prototypes of three poxvirus genera--orthopoxvirus (OPV), parapoxvirus (PPV), avipoxvirus (APV)--and Newcastle disease **virus** (NDV) as a control, as well as three **recombinant** OPV strains and one **recombinant** APV strain, were incubated in vitro with peripheral blood mononuclear leukocytes (PBML) of man, sheep and swine. Antiviral activity was determined in PBML culture supernatants at different time intervals after **virus** cell interaction using a cytopathic effect inhibition bioassay. Additionally, supernatants derived from human PBML were screened for interferons (IFN) alpha and gamma as well as for tumor necrosis factor by enzyme-linked immunosorbent assay. IFN titers reached a maximum 24 h after PBML stimulation at a multiplicity of infection (MOI) greater than 1. IFN alpha/beta was found to be responsible for the antiviral effect. Using a MOI > or = 1 the highly attenuated strain **MVA** was the only representant of vaccinia **virus** (VV) that induced significant amounts of IFN also as a lacZ **recombinant** . Replicable **virus** from five well-known VV strains as well as the Chinese VV strain Tien Tan (VVTT) as a **recombinant** vaccine failed to induce leukocyte IFN. Inactivated VV strain Elstree and the **recombinant** TT strain induced high titers of leukocyte IFN. Supernatants derived from human, porcine and ovine PBML stimulated with replicable PPV, native VV **MVA** and **MVA** lacZ **recombinant** or native APV and APV lacZ **recombinant** **virus** regularly contained IFN alpha. In contrast to NDV, neither specific antisera nor monoclonal antibodies were able to block the INF induction by VV and PPV.

L2 ANSWER 56 OF 63 MEDLINE DUPLICATE 37
AN 95313355 MEDLINE
DN 95313355
TI Replication-deficient vaccinia **virus** encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells.

AU Wyatt L S; Moss B; Rozenblatt S
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0455, USA.
SO VIROLOGY, (1995 Jun 20) 210 (1) 202-5.
Journal code: XEA. ISSN: 0042-6822.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199509
AB The vaccinia **virus**/bacteriophage T7 hybrid transient expression system employs a **recombinant** vaccinia **virus** that encodes the T7 RNA polymerase gene, a plasmid vector with a gene of interest regulated by a T7 promoter, and any cell line suitable for infection and transfection. Although high expression in a majority of cells is achieved, the severe cytopathic effects of vaccinia **virus** and the safety precautions required for use of infectious agents are undesirable features of the system. Here, we report the construction of a highly attenuated and avian host-restricted vaccinia **virus** **recombinant** that encodes the T7 RNA polymerase gene (**MVA** /T7 pol) and demonstrate the use of the **virus** for transient expression in mammalian cells. **MVA**/T7 pol has reduced cytopathic effects compared to the previously used replication-competent vaccinia **virus**, while providing a high level of gene expression in multiple mammalian cell lines.

L2 ANSWER 57 OF 63 MEDLINE DUPLICATE 38
AN 95394156 MEDLINE
DN 95394156
TI Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase.
AU Sutter G; Ohlmann M; Erfle V
CS Institut fur Molekulare Virologie, GSF-Forschungszentrum fur Unwelt und Gesundheit GmbH, Oberschleissheim, FRG.
SO FEBS LETTERS, (1995 Aug 28) 371 (1) 9-12.
Journal code: EUH. ISSN: 0014-5793.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199512
AB Modified vaccinia **virus** **Ankara** (**MVA**), a host range restricted and highly attenuated vaccinia **virus** strain, is unable to multiply in human and most other mammalian cell lines. Since viral gene expression is unimpaired in non-permissive cells **recombinant** **MVA** viruses are efficient as well as exceptionally safe expression vectors. We constructed a **recombinant** **MVA** that expresses the bacteriophage T7 RNA polymerase and tested its usefulness for transient expression of **recombinant** genes under the control of a T7 promoter. Using the chloramphenicol acetyltransferase (CAT) gene as a reporter gene, infection with **MVA**-T7pol allowed efficient synthesis of **recombinant** enzyme in mammalian cells. Despite the severe host restriction of **MVA**, enzyme activities induced by infection with **MVA**-T7pol were similar to those determined after infection with a replication-competent vaccinia-T7pol **recombinant** **virus** . Thus, **MVA**-T7pol may be used as a novel vaccinia vector to achieve T7 RNA polymerase-specific **recombinant** gene expression in the absence of productive vaccinia **virus** replication.

L2 ANSWER 58 OF 63 MEDLINE DUPLICATE 39
AN 95066322 MEDLINE
DN 95066322
TI A **recombinant** vector derived from the host range-restricted and

highly attenuated **MVA** strain of vaccinia **virus** stimulates protective immunity in mice to influenza **virus**.

AU Sutter G; Wyatt L S; Foley P L; Bennink J R; Moss B

CS Laboratory of Viral Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

SO VACCINE, (1994 Aug) 12 (11) 1032-40.
Journal code: X6O. ISSN: 0264-410X.

CY ENGLAND: United Kingdom

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199502

AB The immunogenicity of a **recombinant virus** derived from modified vaccinia **virus Ankara** (**MVA**), a host range-restricted, highly attenuated and safety-tested strain, was investigated. Plasmid transfer vectors that provide strong synthetic early/late promoters for the simultaneous expression of two genes as well as a transient or stable selectable marker and flanking sequences for homologous recombination with the **MVA** genome were constructed. A **recombinant MVA** containing influenza **virus** haemagglutinin and nucleoprotein genes was isolated in avian cells and shown to express both proteins efficiently upon infection of human or mouse cells in which abortive replication occurs. Mice, inoculated by various routes with **recombinant MVA**, produced antibody and cytotoxic T-lymphocyte responses to influenza **virus** proteins and were protected against a lethal influenza **virus** challenge as effectively as mice immunized with a **recombinant** derived from the replication-competent WR strain of vaccinia **virus**.

L2 ANSWER 59 OF 63 MEDLINE DUPLICATE 40

AN 94233740 MEDLINE

DN 94233740

TI Epitope detection in the envelope of intracellular naked orthopox viruses and identification of encoding genes.

AU Czerny C P; Johann S; Holzle L; Meyer H

CS Institute of Medical Microbiology, Infectious and Epidemic Diseases, Veterinary Faculty, Ludwig-Maximillans-University, Munich, Germany..

SO VIROLOGY, (1994 May 1) 200 (2) 764-77.
Journal code: XEA. ISSN: 0042-6822.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals; Cancer Journals

EM 199408

AB Monoclonal antibodies (MAbs) were generated against vaccinia **virus**, cowpox **virus** KR2 Brighton, monkeypox **virus** Copenhagen, or ectromelia **virus**. Pairwise epitope specificity studies by competition ELISAs identified 23 distinct antigenic sites in 19 different orthopox **virus** strains. Six epitopes were completely independent of each other, and 17 closely related antigenic sites formed three separate epitope complexes. As shown by immunogold electron microscopy (ELMI), all MAbs reacted with epitopes in the envelope of intracellular naked **virus**, 16 MAbs recognized proteins of 32, 30, 16 or 14 kDa in Western blotting (WB), and 9 MAbs neutralized **virus** infectivity. In rabbitpox **virus** (RPV) 18 epitopes were detected. A lambda gt11 expression library of RPV DNA was screened with the corresponding 18 MAbs. Fourteen **recombinant** bacteriophage clones (ph) were isolated. Cross-hybridizations of phage and RPV DNA demonstrated a reaction with the HindIII A, HindIII D, or HindIII H fragments, respectively. DNA of ph3D was related to the A25L gene, which corresponds to the A-type inclusion body gene of cowpox **virus**. Two phage clones contained sequences of the 14-kDa fusion protein gene (A27L gene). Ph1A contained nearly the entire 14-kDa gene encoding 4 neutralizing (neutr) and 2 nonneutr epitopes. Ph5, expressing only half of this gene product, encoded 1 nonneutr epitope. The fusion protein of vaccinia **virus MVA** was isolated by immune-affinity

chromatography with a neutr. catching MAb. The protein formed hollow rods (ELMI) and the 6 antigenic sites that were present were identical to those expressed by Escherichia coli infected with ph1A. WB detection with a polyclonal hyperimmune serum detected protein bands of 54, 32, 30, 16, and 14 kDa. The catching MAb bound only to a 16-kDa band. The purified fusion protein induced neutralizing antibodies in mice and rabbits.

L2 ANSWER 60 OF 63 SCISEARCH COPYRIGHT 2000 ISI (R)
AN 93:454256 SCISEARCH
GA The Genuine Article (R) Number: LM946
TI DETECTION OF A PROTEIN ENCODED BY THE VACCINIA **VIRUS** C7L OPEN READING FRAME AND STUDY OF ITS EFFECT ON **VIRUS** MULTIPLICATION IN DIFFERENT CELL-LINES
AU OGUIURA N; SPEHNER D; DRILLIEN R (Reprint)
CS FAC MED STRASBOURG, INSERM, U74, 3 RUE KOEBERLE, F-67000 STRASBOURG, FRANCE; FAC MED STRASBOURG, INST VIROL, ULP, SYNTHELABO, COMMUN LAB, F-67000 STRASBOURG, FRANCE
CYA FRANCE
SO JOURNAL OF GENERAL VIROLOGY, (JUL 1993) Vol. 74, Part 7, pp. 1409-1413. ISSN: 0022-1317.
DT Note; Journal
FS LIFE
LA ENGLISH
REC Reference Count: 24
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB Vaccinia **virus** encodes several proteins, the activity of which is essential for multiplication in different cell types. Both the C7L and K1L open reading frames (ORFs) have been characterized as viral determinants for multiplication in human cells. To confirm and extend these findings we inserted the C7L ORF into the genome of a mutant **virus** unable to multiply in human cells and showed that this **virus** recovered its ability to replicate. Deletion of C7L from a wild-type viral genome did not adversely affect **virus** multiplication in human cells but it did reduce replication in hamster Dede cells. When both C7L and K1L were deleted from the vaccinia **virus** genome only poor or no viral yields were obtained from various human cell lines. **Recombinant** viruses were also constructed to facilitate the study of C7L protein synthesis during infection. One **virus** in which the lacZ ORF was fused downstream and in-frame with the C7L ORF enabled us to characterize the C7L protein as an early gene product. Another **recombinant virus** was constructed so that the carboxy terminus of the C7L ORF product contained an additional 28 amino acids from the carboxy terminus of K1L. Tagging of C7L in this way allowed us to detect the fusion protein by immunoprecipitation with antibodies against the K1L protein. Furthermore, the hybrid protein retained its biological properties. The **recombinant** viruses constructed in this work should be useful for studies of the molecular basis of the activity of viral host range proteins.

L2 ANSWER 61 OF 63 MEDLINE DUPLICATE 41
AN 93066340 MEDLINE
DN 93066340
TI Nonreplicating vaccinia vector efficiently expresses **recombinant** genes.
AU Sutter G; Moss B
CS Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1992 Nov 15) 89 (22) 10847-51.
Journal code: PV3. ISSN: 0027-8424.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; Cancer Journals
EM 199302

AB Modified vaccinia **Ankara** (**MVA**), a highly attenuated vaccinia **virus** strain that has been safety tested in humans, was evaluated for use as an expression vector. **MVA** has multiple genomic deletions and is severely host cell restricted: it grows well in avian cells but is unable to multiply in human and most other mammalian cells tested. Nevertheless, we found that replication of viral DNA appeared normal and that both early and late viral proteins were synthesized in human cells. Proteolytic processing of viral structural proteins was inhibited, however, and only immature **virus** particles were detected by electron microscopy. We constructed an insertion plasmid with the Escherichia coli lacZ gene under the control of the vaccinia **virus** late promoter P11, flanked by sequences of **MVA** DNA, to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the **MVA** genome. **MVA** recombinants were isolated and propagated in permissive avian cells and shown to express the enzyme beta-galactosidase upon infection of nonpermissive human cells. The amount of enzyme made was similar to that produced by a **recombinant** of vaccinia **virus** strain Western Reserve, which also had the lacZ gene under control of the P11 promoter, but multiplied to high titers. Since **recombinant** gene expression is unimpaired in nonpermissive human cells, **MVA** may serve as a highly efficient and exceptionally safe vector.

L2 ANSWER 62 OF 63 JAPIO COPYRIGHT 2000 JPO
AN 1990-005860 JAPIO
TI **RECOMBINANT** VACCINIA **VIRUS MVA**
IN UERUNAA ARUTENBURUGAA
PA F HOFFMANN LA ROCHE & CO AG, CH (CO 000944)
PI JP 02005860 A 19900110 Heisei
AI JP1989-3875 (JP01003875 Heisei) 19890112
PRAI CH 1988-85 19880112

L2 ANSWER 63 OF 63 CAPLUS COPYRIGHT 2000 ACS
AN 1990:104837 CAPLUS
DN 112:104837
TI Use of **recombinant** vaccinia **virus** producing foreign antigens as a vaccine
IN Altenburger, Werner
PA Hoffmann-La Roche, F., und Co. A.-G., Switz.
SO Eur. Pat. Appl., 12 pp.
CODEN: EPXXDW
DT Patent
LA German
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	EP 324350	A1	19890719	EP 1989-100038	19890103
	EP 324350	B1	19931110		
	R: AT, BE, CH, DE, ES, FR, GB, IT, LI, LU, NL, SE				
	AT 97164	E	19931115	AT 1989-100038	19890103
	ES 2059565	T3	19941116	ES 1989-100038	19890103
	US 5185146	A	19930209	US 1989-293738	19890104
	AU 8927743	A1	19890720	AU 1989-27743	19890105
	AU 619235	B2	19920123		
	ZA 8900097	A	19890927	ZA 1989-97	19890105
	DK 8900117	A	19890713	DK 1989-117	19890111
	JP 02005860	A2	19900110	JP 1989-3875	19890112
	JP 2589797	B2	19970312		
PRAI	CH 1988-85		19880112		
	EP 1989-100038		19890103		

AB **Recombinant** vaccinia **virus MVA** or WR prepd. by in vivo homologous recombination with plasmids contg. genes for foreign antigens are used to express these antigens in cell culture or in mice. Tissue culture cells (CV1) were transformed with plasmid pHGS-2/5.1 carrying the gene for a malaria antigen 5.1 and the plaque non-forming

vaccinia **virus MVA** or WR. Recombinants (plaque-forming) were recovered and the **virus** amplified. Tissue culture cells infected with these recombinants produced antigen detectable by immunofluorescence. Mice inoculated with recombinants produced antigen at titers of 1:6,700 (**MVA**) or 1:22,800 (WR). The recombinants were no more neurovirulent than the parent strains in mice.

=> log h

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	69.92	70.07

DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)	SINCE FILE ENTRY	TOTAL SESSION
CA SUBSCRIBER PRICE	-7.24	-7.24

SESSION WILL BE HELD FOR 60 MINUTES
STN INTERNATIONAL SESSION SUSPENDED AT 12:39:13 ON 28 FEB 2000

L2 ANSWER 1 OF 18 MEDLINE
ACCESSION NUMBER: 2001496493 MEDLINE
DOCUMENT NUMBER: 21405825 PubMed ID: 11514732
TITLE: Induction of simian immunodeficiency virus (SIV)-specific CTL in rhesus macaques by vaccination with modified vaccinia virus Ankara expressing SIV transgenes: influence of pre-existing anti-vector immunity.
AUTHOR: Sharpe S; Polyanskaya N; Dennis M; **Sutter G**; Hanke T; Erfle V; Hirsch V; Cranage M
CORPORATE SOURCE: Centre for Applied Microbiology and Research (CAMR), Salisbury SP4 0JG, UK.. sally.sharpe@camr.org.uk
SOURCE: JOURNAL OF GENERAL VIROLOGY, (2001 Sep) 82 (Pt 9) 2215-23. Journal code: I9B; 0077340. ISSN: 0022-1317.
PUB. COUNTRY: England: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200109
ENTRY DATE: Entered STN: 20010910
Last Updated on STN: 20010924
Entered Medline: 20010920

AB A major aim in AIDS vaccine development is the definition of strategies to stimulate strong and durable cytotoxic T lymphocyte (CTL) responses. Here we report that simian immunodeficiency virus (SIV)-specific CTL developed in 4/4 macaques following a single intramuscular injection of modified vaccinia virus Ankara (**MVA**) constructs expressing both structural and regulatory/accessory genes of SIV. In two animals Nef-specific responses persisted, but other responses diminished and new responses were not revealed, following further vaccination. Vaccination of another two macaques, expressing Mamu A*01 MHC class I, with **MVA** constructs containing nef and gag-pol under the control of the moderate strength natural vaccinia virus early/late promoter P7.5, again induced an early Nef-specific response, whereas responses to Gag remained undetectable. Anti-vector immunity induced by this immunization was shown to prevent the efficient stimulation of CTL directed to the cognate Gag epitope, p11C C-M, following vaccination with another **MVA** construct expressing SIV Gag-Pol under a strong synthetic vaccinia virus-specific promoter. In contrast, vaccination of a previously unexposed animal resulted in a SIV-specific CTL response widely disseminated in lymphoid tissues including lymph nodes associated with the rectal and genital routes of SIV entry. Thus, despite the highly attenuated nature of **MVA**, repeated immunization may elicit sufficient anti-vector immunity to limit the effectiveness of later vaccination.

L2 ANSWER 2 OF 18 MEDLINE
ACCESSION NUMBER: 2001494964 MEDLINE
DOCUMENT NUMBER: 21247203 PubMed ID: 11348720
TITLE: Enhanced simian immunodeficiency virus-specific immune responses in macaques induced by priming with recombinant Semliki Forest virus and boosting with modified vaccinia virus Ankara.
AUTHOR: Nilsson C; Makitalo B; Berglund P; Bex F; Liljestrom P; **Sutter G**; Erfle V; ten Haaft P; Heeney J; Biberfeld G; Thorstensson R
CORPORATE SOURCE: Swedish Institute for Infectious Disease Control, SE-171 82, Solna, Sweden.. charlotta.nilsson@smi.ki.se
SOURCE: VACCINE, (2001 May 14) 19 (25-26) 3526-36. Journal code: X60; 8406899. ISSN: 0264-410X.
PUB. COUNTRY: England: United Kingdom
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200109
ENTRY DATE: Entered STN: 20010910
Last Updated on STN: 20010910
Entered Medline: 20010906

AB The immunogenicity of two vector-based vaccines, either given alone or in a prime-boost regimen, was investigated. Cynomolgus macaques were immunised with modified vaccinia virus Ankara (**MVA**) expressing simian immunodeficiency virus (SIV)macJ5 env, gag-pol, nef, rev, and tat genes (**MVA**-SIVmac) or primed with a Semliki forest virus (SFV) vaccine expressing the same genes (SFV-SIVmac) and boosted with **MVA**-SIVmac. Generally, antibody responses, T-cell proliferative responses and cytotoxic T-cell responses remained low or undetectable in vaccinees receiving **MVA**-SIVmac or SFV-SIVmac alone. In contrast, monkeys who first received SFV-SIVmac twice and then were boosted with **MVA**-SIVmac showed increased antibody responses as well as high T-cell proliferative responses. Three of these vaccinees had cytotoxic T-lymphocytes directed against three or four of the gene products. No evidence of protection was seen against an intrarectal heterologous SIVsm challenge given 3 months after the last immunisation. The study demonstrates a prime-boost strategy that efficiently induces both humoral and cellular immune responses.

L2 ANSWER 3 OF 18 MEDLINE
ACCESSION NUMBER: 2001355178 MEDLINE
DOCUMENT NUMBER: 21179862 PubMed ID: 11282186
TITLE: Vaccination with recombinant modified vaccinia virus Ankara protects against measles virus infection in the mouse and cotton rat model.
AUTHOR: Weidinger G; Ohlmann M; Schlereth B; **Sutter G**; Niewiesk S
CORPORATE SOURCE: Institute of Virology and Immunobiology, University of Wuerzburg, Versbacher Str. 7, 97078 Wurzburg, Germany.
SOURCE: VACCINE, (2001 Apr 6) 19 (20-22) 2764-8.
Journal code: X6O; 8406899. ISSN: 0264-410X.
PUB. COUNTRY: England: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200106
ENTRY DATE: Entered STN: 20010625
Last Updated on STN: 20010625
Entered Medline: 20010621

AB Modified vaccinia virus Ankara (**MVA**) has been used as an experimental vaccine vector against respiratory infections. We have tested the safety and immunogenicity of a recombinant virus expressing the hemagglutinin of measles virus (**MVA**-MV-H) using the mouse model of measles virus induced encephalitis and the cotton rat model for respiratory infection. **MVA**-MV-H proved to induce a TH1 response, neutralizing antibodies and conferred protection against both encephalitis and lung infection. The cotton rat is very sensitive to infection with replication competent vaccinia virus. In these animals **MVA**-MV-H proved to be a very well tolerated vaccine. However, the efficiency in the presence of MV specific maternal antibodies was low (even using a prime-boost strategy) and therefore might have to be improved.

L2 ANSWER 4 OF 18 MEDLINE
ACCESSION NUMBER: 2000507928 MEDLINE
DOCUMENT NUMBER: 20511539 PubMed ID: 11054673
TITLE: Transporter (TAP)- and proteasome-independent presentation of a melanoma-associated tyrosinase epitope.
AUTHOR: Wolfel C; Drexler I; Van Pel A; Thres T; Leister N; Herr W; **Sutter G**; Huber C; Wolfel T
CORPORATE SOURCE: III. Medizinische Klinik, Johannes Gutenberg-Universitat, Mainz, Germany.. t.woelfel@3-med.klinik.uni-mainz.de

SOURCE: INTERNATIONAL JOURNAL OF CANCER, (2000 Nov 1) 88 (3) 432-8.
Journal code: GQU. ISSN: 0020-7136.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200011
ENTRY DATE: Entered STN: 20010322
Last Updated on STN: 20010322
Entered Medline: 20001113

AB The melanosomal protein tyrosinase is considered as a target of specific immunotherapy against melanoma. Two tyrosinase-derived peptides are presented in association with HLA-A2.1 [Wolfel et al., Eur. J. Immunol., 24, 759-764 (1994)]. Peptide 1-9 (MLLAVLYCL) is generated from the putative signal sequence. The internal peptide 369-377 is posttranslationally converted at residue 371, and its presentation is dependent on functional TAP transporters and proteasomes [Mosse et al., J. exp. Med.187, 37-48 (1998)]. Herein, we report on the processing and transport requirements for the signal sequence-derived peptide 1-9 that were studied in parallel to those for peptide 369-377. After infection of TAP-deficient (T2) and TAP-positive (T1) cells with a Modified Vaccinia Ankara construct carrying the human tyrosinase gene (**MVA**-hTyr), we found that recognition by CTL against peptide 1-9 did not require TAP function as opposed to recognition by CTL against peptide 369-377. When target cells with intact processing and transport functions were infected with **MVA**-hTyr, lysis by CTL against peptide 1-9 was not impaired by lactacystin, a specific inhibitor for the proteasome, whereas lysis by CTL against peptide 369-377 was completely abrogated. Taken together, peptide 1-9 derived from the signal sequence of tyrosinase is presented in a TAP-independent fashion and does not require proteasomes for processing. Cellular immune responses against this hydrophobic peptide can be monitored with lymphokine spot assays as documented in the case of a patient with metastatic melanoma, in whom we observed a preferential T-cell response against tyrosinase peptide 1-9 subsequent to chemoimmunotherapy. Independence of cytosolic processing and transport pathways and potentially enhanced expression levels make signal sequence-derived peptides and their carrier proteins important candidates for specific immunotherapy.

L2 ANSWER 5 OF 18 MEDLINE
ACCESSION NUMBER: 2000492050 MEDLINE
DOCUMENT NUMBER: 20326261 PubMed ID: 10868279
TITLE: Transient host range selection for genetic engineering of modified vaccinia virus Ankara.
AUTHOR: Staib C; Drexler I; Ohlmann M; Wintersperger S; Erfle V; **Sutter G**
CORPORATE SOURCE: GSF-Institute for Molecular Virology, Munich, Germany.
SOURCE: BIOTECHNIQUES, (2000 Jun) 28 (6) 1137-42, 1144-6, 1148.
Journal code: AN3; 8306785. ISSN: 0736-6205.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200010
ENTRY DATE: Entered STN: 20001027
Last Updated on STN: 20001027
Entered Medline: 20001019

AB Recombinant vaccinia viruses are extremely valuable tools for research in molecular biology and immunology. The extension of vaccinia vector technology to replication-deficient and safety-tested virus strains such as modified vaccinia virus Ankara (**MVA**) have made this versatile eukaryotic expression system even more attractive for basic and clinical research. Here, we report on easily obtaining recombinant **MVA** using stringent growth selection on rabbit kidney RK-13 cells. We describe the construction and use of new **MVA** vector plasmids that carry an expression cassette of the vaccinia virus host range gene, K1L, as a

transient selectable marker. These plasmids allow either stable insertion of additional recombinant genes into the **MVA** genome or precisely targeted mutagenesis of **MVA** genomic sequences. Repetitive DNA sequences flanking the K1L gene were designed to remove the marker gene from the viral genome by homologous recombination under nonselective growth conditions. The convenience of this new selection technique is demonstrated by isolating **MVA** recombinants that produce green fluorescent protein and by generating **MVA** deletion mutants.

L2 ANSWER 6 OF 18 MEDLINE
ACCESSION NUMBER: 2000204208 MEDLINE
DOCUMENT NUMBER: 20204208 PubMed ID: 10738224
TITLE: Human tumor growth is inhibited by a vaccinia virus carrying the E2 gene of bovine papillomavirus.
AUTHOR: Valdez Graham V; **Sutter G**; Jose M V; Garcia-Carranca A; Erfle V; Moreno Mendoza N; Merchant H; Rosales R
CORPORATE SOURCE: Department of Molecular Biology, Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico.
SOURCE: CANCER, (2000 Apr 1) 88 (7) 1650-62.
Journal code: CLZ; 0374236. ISSN: 0008-543X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 200004
ENTRY DATE: Entered STN: 20000505
Last Updated on STN: 20000505
Entered Medline: 20000427

AB BACKGROUND: Papillomavirus is the etiologic agent associated with cervical carcinoma. The papilloma E2 protein is able to regulate negatively the expression of E6 and E7 papilloma oncoproteins. Therefore, a new, highly attenuated vaccinia virus known as modified vaccinia virus Ankara (**MVA**), which carries the papillomavirus E2 gene, was used for the treatment of tumors associated with human papillomavirus. METHODS: Analysis of expression of the E2 gene from the recombinant vaccinia virus was performed by reverse transcription-polymerase chain reaction of RNA isolated from infected cells. Detection of the E2 protein was done by immunoprecipitation from proteins labeled with [(35)S]-methionine, isolated from infected cells. The therapeutic effect of the **MVA** E2 recombinant virus over human tumors was tested in nude mice bearing tumors generated by inoculation of HeLa cells. Series of 10 nude mice with tumors of different sizes were injected with **MVA**, **MVA** E2, or phosphate-buffered saline. Tumor size was monitored every week to assess growth. RESULTS: The **MVA** E2 recombinant virus efficiently expressed the E2 protein in BS-C-1 cells. This protein was able to repress, in vivo, the papillomavirus P105 promoter, which controls the expression of the E6 and E7 oncoproteins. In nude mice the **MVA** E2 virus reduced tumor growth very efficiently. In contrast, tumors continued to grow in mice treated with **MVA** or PBS. The life expectancy of **MVA** E2-treated mice was also increased three- to fourfold compared with that of animals that received **MVA** or PBS. CONCLUSIONS: The growth of human tumors was efficiently inhibited by the **MVA** E2 recombinant vaccinia virus. The absence of side effects in treated animals suggested that the **MVA** E2 virus is a safe biologic agent that could in the future be used in humans for the treatment of cervical carcinoma.

L2 ANSWER 7 OF 18 MEDLINE
ACCESSION NUMBER: 1999446898 MEDLINE
DOCUMENT NUMBER: 99446898 PubMed ID: 10519409
TITLE: Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen

A*0201-restricted cytotoxic T cells in vitro and in vivo.
AUTHOR: Drexler I; Antunes E; Schmitz M; Wolfel T; Huber C; Erfle V; Rieber P; Theobald M; **Sutter G**
CORPORATE SOURCE: GSF-Institute for Molecular Virology, Munich, Germany.. drexler@gsf.de
SOURCE: CANCER RESEARCH, (1999 Oct 1) 59 (19) 4955-63.
Journal code: CNF; 2984705R. ISSN: 0008-5472.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199911
ENTRY DATE: Entered STN: 20000111
Last Updated on STN: 20000111
Entered Medline: 19991104

AB Vaccination with tumor-associated antigens is a promising approach for cancer immunotherapy. Because the majority of these antigens are normal self antigens, they may require suitable delivery systems to promote their immunogenicity. A recombinant vector based on the modified vaccinia virus Ankara (**MVA**) was used for expression of human tyrosinase, a melanoma-specific differentiation antigen, and evaluated for its efficacy as an antitumor vaccine. Stable recombinant viruses (**MVA**-hTyr) were constructed that have deleted the selection marker lacZ and efficiently expressed human tyrosinase in primary human cells and cell lines. Tyrosinase-specific human CTLs were activated in vitro by **MVA**-hTyr-infected, HLA-A*0201-positive human dendritic cells. Importantly, an efficient tyrosinase- and melanoma-specific CTL response was induced in vitro using **MVA**-hTyr-infected autologous dendritic cells as activators for peripheral blood mononuclear cells derived from HLA-A*0201-positive melanoma patients despite prior vaccination against smallpox. Immunization of HLA-A*0201/Kb transgenic mice with **MVA**-hTyr induced A*0201-restricted CTLs specific for the human tyrosinase-derived peptide epitope 369-377. These in vivo primed CTLs were of sufficiently high avidity to recognize and lyse human melanoma cells, which present the endogenously processed tyrosinase peptide in the context of A*0201. Tyrosinase-specific CTL responses were significantly augmented by repeated vaccination with **MVA**-hTyr. These findings demonstrate that HLA-restricted CTLs specific for human tumor-associated antigens can be efficiently generated by immunization with recombinant **MVA** vaccines. The results are an essential basis for **MVA**-based vaccination trials in cancer patients.

L2 ANSWER 8 OF 18 MEDLINE
ACCESSION NUMBER: 1999114127 MEDLINE
DOCUMENT NUMBER: 99114127 PubMed ID: 9917089
TITLE: Gene transfer into human dendritic antigen-presenting cells by vaccinia virus and adenovirus vectors.
AUTHOR: Di Nicola M; Siena S; Bregni M; Longoni P; Magni M; Milanesi M; Matteucci P; Mortarini R; Anichini A; Parmiani G; Drexler I; Erfle V; **Sutter G**; Gianni A M
CORPORATE SOURCE: Division of Medical Oncology (OMC), Istituto Nazionale Tumori, Milan, Italy.
SOURCE: CANCER GENE THERAPY, (1998 Nov-Dec) 5 (6) 350-6.
Journal code: CE3; 9432230. ISSN: 0929-1903.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199903
ENTRY DATE: Entered STN: 19990402
Last Updated on STN: 19990402
Entered Medline: 19990322

AB In a search for means to deliver exogenous gene(s) into human dendritic cells (DCs) from the perspective of tumor-specific vaccination, we have evaluated two recombinant viruses, both of which carry a reporter gene which is namely a modified vaccinia virus Ankara (**MVA**) and an

adenovirus, as possible expression vectors. The recombinant **MVA**-P11 LZ vector carries the Escherichia coli lacZ gene coding for the enzyme beta-galactosidase, and the recombinant Ad-MFG-AP vector carries a modified membrane-exposed alkaline phosphatase (AP) gene. DCs were generated ex vivo in the presence of tumor necrosis factor-alpha, granulocyte macrophage colony-stimulating factor, stem cell factor, and flk-2/flt-3 ligand taken from CD34+ hematopoietic progenitors that were mobilized into the peripheral blood of cancer patients treated with high-dose cyclophosphamide and filgrastim. The target cells used for gene delivery were either CD34+ cells that had been subsequently induced to differentiate into mature DCs or DCs transduced after ex vivo generation from CD34+ cells. The results showed that: (a) infection of CD34+ cell derived-DCs (mature DCs) with either viral vector resulted in the efficient synthesis of recombinant protein, and (b) CD34+ cells were permissive for the expression of the recombinant reporter gene after infection with Ad-MFG-AP but not after infection with **MVA**-P11 LZ. In conclusion, these results suggest that vaccinia and adenovirus vectors are candidate to act as vehicles in genetically engineering human DCs.

L2 ANSWER 9 OF 18 MEDLINE
ACCESSION NUMBER: 1999102651 MEDLINE
DOCUMENT NUMBER: 99102651 PubMed ID: 9847398
TITLE: Feline calicivirus capsid protein expression and capsid assembly in cultured feline cells.
AUTHOR: Geissler K; Schneider K; Fleuchaus A; Parrish C R; **Sutter G**; Truyen U
CORPORATE SOURCE: Institute for Medical Microbiology, Infectious and Epidemic Diseases, Ludwig Maximilians University Munich, 80539 Munich, Germany.
SOURCE: JOURNAL OF VIROLOGY, (1999 Jan) 73 (1) 834-8.
Journal code: KCV; 0113724. ISSN: 0022-538X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199901
ENTRY DATE: Entered STN: 19990209
Last Updated on STN: 19990209
Entered Medline: 19990128

AB The capsid protein of feline calicivirus (FCV) was expressed by using plasmids containing cytomegalovirus, simian virus 40, or T7 promoters. The strongest expression was achieved with the T7 promoter and coinfection with vaccinia virus expressing the T7 RNA polymerase (**MVA**/T7pol). The FCV precursor capsid protein was processed to the mature-size protein, and these proteins were assembled in to virus-like particles.

L2 ANSWER 10 OF 18 MEDLINE
ACCESSION NUMBER: 1998131961 MEDLINE
DOCUMENT NUMBER: 98131961 PubMed ID: 9472619
TITLE: Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells.
AUTHOR: Drexler I; Heller K; Wahren B; Erfle V; **Sutter G**
CORPORATE SOURCE: Institut fur Molekulare Virologie, GSF-Forschungszentrum fur Umwelt und Gesundheit GmbH, Munchen-Neuherberg, Germany.
SOURCE: JOURNAL OF GENERAL VIROLOGY, (1998 Feb) 79 (Pt 2) 347-52.
Journal code: I9B; 0077340. ISSN: 0022-1317.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199802
ENTRY DATE: Entered STN: 19980306

Last Updated on STN: 19980306
Entered Medline: 19980226

AB Although desirable for safety reasons, the host range restrictions of modified vaccinia virus Ankara (**MVA**) make it less applicable for general use. Propagation in primary chicken embryo fibroblasts (CEF) requires particular cell culture experience and has no pre-established record of tissue culture reproducibility. We investigated a variety of established cell lines for productive virus growth and recombinant gene expression. Baby hamster kidney cells (BHK), a well-characterized, easily maintained cell line, supported **MVA** growth and as proficient expression of the E. coli lacZ reporter gene as the highly efficient CEF, whereas other cell lines were non-permissive or allowed only very limited **MVA** replication. Importantly, no virus production occurred in patient-derived infected primary human cells. These results emphasize the safety and now improved accessibility of **MVA** for the development of expression vectors and live recombinant vaccines.

L2 ANSWER 11 OF 18 MEDLINE
ACCESSION NUMBER: 97181369 MEDLINE
DOCUMENT NUMBER: 97181369 PubMed ID: 9029530
TITLE: Rescue of measles virus using a replication-deficient vaccinia-T7 vector.
AUTHOR: Schneider H; Spielhofer P; Kaelin K; Dotsch C; Radecke F; **Sutter G**; Billeter M A
CORPORATE SOURCE: Institut fur Molekularbiologie I, Universitat Zurich, Switzerland.
CONTRACT NUMBER: 5 R01 AI35136 (NIAID)
SOURCE: JOURNAL OF VIROLOGICAL METHODS, (1997 Feb) 64 (1) 57-64. Journal code: HQR; 8005839. ISSN: 0166-0934.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-Z66517
ENTRY MONTH: 199704
ENTRY DATE: Entered STN: 19970424
Last Updated on STN: 19980206
Entered Medline: 19970417

AB A system which allows the reconstitution of measles virus (MV) from cloned cDNA is described. The severely host cell restricted vaccinia vector **MVA**-T7 expressing bacteriophage T7 RNA polymerase was used to generate full-length antigenomic MV RNA and simultaneously the mRNAs encoding the viral N, P and L proteins in order to produce replicationally and transcriptionally active nucleocapsids. The functionality of the N, P and L proteins was demonstrated first by their ability to rescue MV specific subgenomic RNAs. Assembly and budding of reconstituted MV was shown by syncytia formation and subsequently by virus isolation. The inability of **MVA**-T7 to produce progeny virus in most mammalian cells circumvents the necessity to separate the reconstituted MV from the **MVA**-T7 helper virus. Since all components are expressed transiently, this system is especially suitable for studying the functions of N, P and L. Furthermore, it is useful for investigating later steps in the MV life cycle.

L2 ANSWER 12 OF 18 MEDLINE
ACCESSION NUMBER: 96342131 MEDLINE
DOCUMENT NUMBER: 96342131 PubMed ID: 8718576
TITLE: Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates.
AUTHOR: Moss B; Carroll M W; Wyatt L S; Bennink J R; Hirsch V M; Goldstein S; Elkins W R; Fuerst T R; Lifson J D; Piatak M; Restifo N P; Overwijk W; Chamberlain R; Rosenberg S A; **Sutter G**
CORPORATE SOURCE: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.

SOURCE: ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, (1996) 397 7-13. Ref: 35
Journal code: 2LU; 0121103. ISSN: 0065-2598.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199701
ENTRY DATE: Entered STN: 19970219
Last Updated on STN: 19970219
Entered Medline: 19970123

AB Three model systems were used to demonstrate the immunogenicity of highly attenuated and replication-defective recombinant **MVA**. (1) Intramuscular inoculation of **MVA**-IN-Fha/np induced humoral and cell-mediated immune responses in mice and protectively immunized them against a lethal respiratory challenge with influenza virus. Intranasal vaccination was also protective, although higher doses were needed. (2) In rhesus macaques, an immunization scheme involving intramuscular injections of **MVA**-SIVenv/gag/pol greatly reduced the severity of disease caused by an SIV challenge. (3) In a murine cancer model, immunization with **MVA**-beta gal prevented the establishment of tumor metastases and even prolonged life in animals with established tumors. These results, together with previous data on the safety of **MVA** in humans, suggest the potential usefulness of recombinant **MVA** for prophylactic vaccination and therapeutic treatment of infectious diseases and cancer.

L2 ANSWER 13 OF 18 MEDLINE
ACCESSION NUMBER: 95394156 MEDLINE
DOCUMENT NUMBER: 95394156 PubMed ID: 7664891
TITLE: Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase.
AUTHOR: **Sutter G**; Ohlmann M; Erfle V
CORPORATE SOURCE: Institut fur Molekulare Virologie, GSF-Forschungszentrum fur Unwelt und Gesundheit GmbH, Oberschleissheim, FRG.
SOURCE: FEBS LETTERS, (1995 Aug 28) 371 (1) 9-12.
Journal code: EUH; 0155157. ISSN: 0014-5793.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199510
ENTRY DATE: Entered STN: 19951020
Last Updated on STN: 19980206
Entered Medline: 19951006

AB Modified vaccinia virus Ankara (**MVA**), a host range restricted and highly attenuated vaccinia virus strain, is unable to multiply in human and most other mammalian cell lines. Since viral gene expression is unimpaired in non-permissive cells recombinant **MVA** viruses are efficient as well as exceptionally safe expression vectors. We constructed a recombinant **MVA** that expresses the bacteriophage T7 RNA polymerase and tested its usefulness for transient expression of recombinant genes under the control of a T7 promoter. Using the chloramphenicol acetyltransferase (CAT) gene as a reporter gene, infection with **MVA**-T7pol allowed efficient synthesis of recombinant enzyme in mammalian cells. Despite the severe host restriction of **MVA**, enzyme activities induced by infection with **MVA**-T7pol were similar to those determined after infection with a replication-competent vaccinia-T7pol recombinant virus. Thus, **MVA**-T7pol may be used as a novel vaccinia vector to achieve T7 RNA polymerase-specific recombinant gene expression in the absence of productive vaccinia virus replication.

L2 ANSWER 14 OF 18 MEDLINE
ACCESSION NUMBER: 95317472 MEDLINE

DOCUMENT NUMBER: 95317472 PubMed ID: 7796954
TITLE: Novel vaccinia vector derived from the host range restricted and highly attenuated **MVA** strain of vaccinia virus.
AUTHOR: **Sutter G**; Moss B
CORPORATE SOURCE: Institute of Molecular Virology, GSF-Centre for Environmental and Health Research, Oberschleissheim, Germany.
SOURCE: DEVELOPMENTS IN BIOLOGICAL STANDARDIZATION, (1995) 84 195-200.
Journal code: E7V; 0427140. ISSN: 0301-5149.
PUB. COUNTRY: Switzerland
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199508
ENTRY DATE: Entered STN: 19950817
Last Updated on STN: 19950817
Entered Medline: 19950801

L2 ANSWER 15 OF 18 MEDLINE
ACCESSION NUMBER: 95066322 MEDLINE
DOCUMENT NUMBER: 95066322 PubMed ID: 7975844
TITLE: A recombinant vector derived from the host range-restricted and highly attenuated **MVA** strain of vaccinia virus stimulates protective immunity in mice to influenza virus.
AUTHOR: **Sutter G**; Wyatt L S; Foley P L; Bennink J R; Moss B
CORPORATE SOURCE: Laboratory of Viral Disease, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
SOURCE: VACCINE, (1994 Aug) 12 (11) 1032-40.
Journal code: X6O; 8406899. ISSN: 0264-410X.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199412
ENTRY DATE: Entered STN: 19950110
Last Updated on STN: 19950110
Entered Medline: 19941216
AB The immunogenicity of a recombinant virus derived from modified vaccinia virus Ankara (**MVA**), a host range-restricted, highly attenuated and safety-tested strain, was investigated. Plasmid transfer vectors that provide strong synthetic early/late promoters for the simultaneous expression of two genes as well as a transient or stable selectable marker and flanking sequences for homologous recombination with the **MVA** genome were constructed. A recombinant **MVA** containing influenza virus haemagglutinin and nucleoprotein genes was isolated in avian cells and shown to express both proteins efficiently upon infection of human or mouse cells in which abortive replication occurs. Mice, inoculated by various routes with recombinant **MVA**, produced antibody and cytotoxic T-lymphocyte responses to influenza virus proteins and were protected against a lethal influenza virus challenge as effectively as mice immunized with a recombinant derived from the replication-competent WR strain of vaccinia virus.

L2 ANSWER 16 OF 18 MEDLINE
ACCESSION NUMBER: 94267870 MEDLINE
DOCUMENT NUMBER: 94267870 PubMed ID: 8207789
TITLE: Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant.
AUTHOR: **Sutter G**; Ramsey-Ewing A; Rosales R; Moss B
CORPORATE SOURCE: Laboratory of Viral Diseases, National Institute of Allergy

and Infectious Diseases, Bethesda, Maryland 20892.
SOURCE: JOURNAL OF VIROLOGY, (1994 Jul) 68 (7) 4109-16.
Journal code: KCV; 0113724. ISSN: 0022-538X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199407
ENTRY DATE: Entered STN: 19940721
Last Updated on STN: 19940721
Entered Medline: 19940713

AB Modified vaccinia virus Ankara (**MVA**), having acquired genomic deletions during passage in chicken embryo fibroblasts, is highly attenuated and unable to productively infect most mammalian cell lines. Multiplication in rabbit kidney-derived RK13 cells, but not other nonpermissive cells, can be restored by insertion of the vaccinia virus K1L gene into the **MVA** genome. During nonproductive infection of RK13 cells by **MVA**, transcription of representative viral early genes was revealed by Northern (RNA) blotting, whereas synthesis of an intermediate mRNA and replication of viral DNA could not be detected. Despite the persistence of viral early mRNA for at least several hours, synthesis of virus-induced polypeptides occurred only during the first hour and was followed by abrupt inhibition of all protein synthesis. Transfection of RK13 cells with a eukaryotic expression plasmid that contained the K1L gene allowed **MVA** infection to proceed to late stages of viral protein synthesis. Moreover, RK13 cell lines that stably expressed the K1L gene were permissive for **MVA** as well as a K1E deletion mutant of the WR strain of vaccinia virus. This is the first description of the complementation of a poxvirus mutant by cells that stably express a viral gene.

L2 ANSWER 17 OF 18 MEDLINE
ACCESSION NUMBER: 93066340 MEDLINE
DOCUMENT NUMBER: 93066340 PubMed ID: 1438287
TITLE: Nonreplicating vaccinia vector efficiently expresses recombinant genes.
AUTHOR: **Sutter G**; Moss B
CORPORATE SOURCE: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1992 Nov 15) 89 (22) 10847-51.
Journal code: PV3; 7505876. ISSN: 0027-8424.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199212
ENTRY DATE: Entered STN: 19930122
Last Updated on STN: 19970203
Entered Medline: 19921223

AB Modified vaccinia Ankara (**MVA**), a highly attenuated vaccinia virus strain that has been safety tested in humans, was evaluated for use as an expression vector. **MVA** has multiple genomic deletions and is severely host cell restricted: it grows well in avian cells but is unable to multiply in human and most other mammalian cells tested. Nevertheless, we found that replication of viral DNA appeared normal and that both early and late viral proteins were synthesized in human cells. Proteolytic processing of viral structural proteins was inhibited, however, and only immature virus particles were detected by electron microscopy. We constructed an insertion plasmid with the Escherichia coli lacZ gene under the control of the vaccinia virus late promoter P11, flanked by sequences of **MVA** DNA, to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the **MVA** genome. **MVA** recombinants were isolated and propagated in permissive avian cells and shown to express the enzyme

beta-galactosidase upon infection of nonpermissive human cells. The amount of enzyme made was similar to that produced by a recombinant of vaccinia virus strain Western Reserve, which also had the lacZ gene under control of the P11 promoter, but multiplied to high titers. Since recombinant gene expression is unimpaired in nonpermissive human cells, **MVA** may serve as a highly efficient and exceptionally safe vector.

L2 ANSWER 18 OF 18 MEDLINE
ACCESSION NUMBER: 91237336 MEDLINE
DOCUMENT NUMBER: 91237336 PubMed ID: 2033387
TITLE: Mapping of deletions in the genome of the highly attenuated vaccinia virus **MVA** and their influence on virulence.
AUTHOR: Meyer H; **Sutter G**; Mayr A
CORPORATE SOURCE: Institute of Medical Microbiology, Infectious and Epidemic Diseases, Veterinary Faculty, Ludwig-Maximilians Universitat, Munchen, Germany.
SOURCE: JOURNAL OF GENERAL VIROLOGY, (1991 May) 72 (Pt 5) 1031-8. Journal code: I9B; 0077340. ISSN: 0022-1317.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199106
ENTRY DATE: Entered STN: 19910714
Last Updated on STN: 19970203
Entered Medline: 19910621

AB Different passages of the vaccinia virus strain Ankara (CVA wild-type) during attenuation to **MVA** (modified vaccinia virus Ankara) have been analysed to detect alterations in the genome. Physical maps for the restriction enzymes HindIII and XhoI have been established. Six major deletions relative to the wild-type strain CVA could be localized. They reduce the size of the entire genome from 208 kb (CVA wild-type) to 177 kb for the **MVA** strain. Four deletions occurred during the first 382 passages and the resulting variant (CVA 382) displays an attenuated phenotype similar to that of the **MVA** strain. The deletions are located in both terminal fragments, affect two-thirds of the host range gene K1L and eliminate 3.5 kb of a highly conserved region in the HindIII A fragment. During the next 190 passages leading to **MVA** two additional deletions appeared. Again, one is located in the left terminal fragment, and the other includes the A-type inclusion body gene. Neither of the deletions appear to participate in further attenuation of the virus. Rescue of the partially deleted host range region with the corresponding wild-type DNA restored the ability of the attenuated strains **MVA** and CVA 382 to grow in some non-permissive tissue cultures. Nevertheless, the complete host range of the wild-type strain was not recovered. Also, plaque-forming behaviour and reduced virulence were not influenced. From the data presented it may be concluded that the partially deleted host range gene is not solely responsible for attenuation.

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Epitope Mapping by Deletion Mutants and Chimeras of Two Vesicular Stomatitis Virus Glycoprotein Genes Expressed by a Vaccinia Virus Vector

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Deletion mutants and chimeras of the glycoprotein (G) genes of vesicular stomatitis virus serotypes Indiana (VSV-Ind) and New Jersey (VSV-NJ) were cloned in plasmids and vaccinia virus vectors under control of the bacteriophage T7 polymerase promoter for expression in CV-1 cells co-infected with a T7 polymerase-expressing vaccinia virus recombinant. Truncated and chimeric G proteins expressed by these vectors were tested for their capacity to react with VSV-Ind and VSV-NJ epitope-specific monoclonal antibodies (MAbs) by Western blot analysis for those antigenic determinants not affected by disulfide-bond reducing conditions or by immuno dotblot analysis for those that are. These experiments allowed us to create putative epitope maps for glycoproteins of both serotypes based on binding affinity and cross-reactivity of VSV-Ind and VSV-NJ MAbs for truncated or chimeric G proteins of known amino acid sequences. Seven of the 9 VSV-NJ G epitopes, including all 4 epitopes involved in virus neutralization by MAbs, mapped to the center (amino acid sequence 193–289) of the 517 amino acid VSV-NJ G protein. Four of the 11 VSV-Ind G epitopes, including 2 neutralizable epitopes, mapped to the cysteine-rich amino-terminal domain (amino acid sequence 80–183) of the 511 amino acid VSV-Ind G protein; the remaining 7 VSV-Ind G epitopes, including 2 involved in virus neutralization, were clustered in the cysteine-poor carboxy-terminal domain (amino acid sequence 286–428). In site-specific mutants of the VSV-Ind G gene defective in one or both glycosylation sites, only the amino-terminal epitopes of the VSV-Ind G protein were affected by deletion of the carbohydrate chain at residue 179; deletion of the carbohydrate chain at residue 336 did not alter reactivity of the G protein with any of the relevant monoclonal antibodies. These results are discussed in relation to earlier attempts to map the antigenic determinants of VSV-NJ and VSV-Ind G proteins by proteolysis of the G protein and by sequencing the G genes of mutant viruses selected for their resistance to neutralization by epitope-specific monoclonal antibodies.

INTRODUCTION

The rhabdovirus, vesicular stomatitis virus (VSV), has been widely used as a model system for studying humoral and cellular immune responses (Wagner, 1987). There, are two major serotypes of VSV, designated Indiana (VSV-Ind) and New Jersey (VSV-NJ). The surface glycoprotein (G) anchored in the membrane of VSV is the type-specific antigen, which gives rise to and reacts with neutralizing antibody (Kelley *et al.*, 1972). The VSV G protein also serves as the major organ for virus adsorption to host cell surface receptors (Bishop *et al.*, 1975) and also mediates virus penetration and uncoating (Matlin *et al.*, 1982). We had previously found by competitive binding studies that MAbs to the VSV-Ind G protein can react with 11 distinct antigenic determinants (epitopes), 4 of which are concerned with neutralization of viral infectivity (Volk *et al.*, 1982). In a similar study, we were able to identify 9 epitopes, at least 4 of which give rise to neutralizing monoclonal antibodies, on the G protein of VSV-NJ (Bricker *et al.*, 1987). Similar results had been reported by Le-Francois and Lyles (1982a,b). Due to the complex secondary and three-dimensional structure of the VSV-NJ G protein, we have been unable to map its antigenic determinants by conventional protease or chemical cleavage techniques. However, a rough estimate of the location of epitopes on the VSV-NJ G protein could be made based on protease footprinting of the G protein partially protected by individual MAbs complexed with staphylococcal protein A–Sepharose beads (Bricker *et al.*, 1987). This technique provided suggestive evidence that a 12-kDa fragment derived from the central fifth of the G protein, extending from amino acid position 219 to approximately 321, is involved in the binding of monoclonal antibodies directed to most, if not all, of the 9 epitopes of the VSV-NJ G protein clustered in this region (Bricker *et al.*, 1987).

Monoclonal antibodies that neutralize viral infectivity have also been used for genetic studies by selecting mutant viruses that resist neutralization. Vandepol *et al.* (1986) have sequenced the cDNAs of G genes of VSV-Ind variants that survived neutralization by the use of monoclonal and polyclonal antibodies. In a similar study, we have used a single short exposure of wild-

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Cells were harvested at 48 hr after infection, lysed, and subjected to 12.5% polyacrylamide–SDS slab gel electrophoresis as described previously (Carroll and Wagner, 1979).

Expression of VSV glycoproteins in cells co-infected with two recombinant vaccinia viruses.

CV-1 cells grown to 80% confluence in 35-mm plates were co-infected with vTF1-6,2 and the recombinant vaccinia virus having the glycoprotein gene of interest flanked by the T7 polymerase promoter and terminator sequences inserted in its TK locus. The multiplicity of infection for each of the recombinants was ~15 PFU/cell. The virus mixture was allowed to adsorb for 2 hr at 37° with occasional rocking of the plate. The residual inoculum was then removed, each plate was washed once with 2 ml of serum-free medium, and 3 ml of MEM with 5% FBS was added. Cells were harvested after 48 hr of incubation at 37°. To obtain large amounts of expressed glycoproteins, the co-transfections or double infections were carried out as described above except that the cells to be infected were cultured in 150-cm^2 tissue-culture flasks. The amounts of virus, plasmid, and media used in the amplified transfection and co-infection procedures were 20-fold greater than that described above.

Western blot and immuno dotblot analysis

The proteins separated on polyacrylamide gels were transferred by electroblotting onto nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH) as described by Towbin *et al.* (1979). The nitrocellulose sheets were then reacted with polyclonal rabbit antiserum or monoclonal antibody followed by incubation with ^{125}I-labeled *Staphylococcus* protein A or by ^{125}I-labeled goat anti-mouse IgG, as previously described in detail (Pal *et al.*, 1985). The monoclonal antibodies used in these studies were prepared from hybridomas made in our laboratory and previously described in detail. (Volk *et al.*, 1982; Bricker *et al.*, 1987). Immuno dotblot analysis was performed 48 hr after infection of CV-1 cells expressing VSV glycoproteins. Aliquots of cells lysed with PBS containing 0.5% NP-40, and containing 30 μg of total protein, were spotted on nitrocellulose sheets and washed with PBS. Dots 0.5 cm in diameter were individually reacted with monoclonal antibody followed by incubation with ^{125}I-labeled goat anti-mouse IgG. In the case of VSV MAbs 1, 7, 11, 12, and VSV-NJ MAbs 15, 19, 20, 22, ^{125}I-labeled *Staphylococcus* protein A was used instead of anti-mouse antibody since lower background was observed under these conditions. After washing, excised nitrocellulose pieces were dissolved in Ready-Solv (Beckman Instruments, Inc., Fullerton, CA) and radioactivity was counted by liquid scintillation spectrometry.

RESULTS

Construction of transient expression vectors and recombinant vaccinia vectors

In order to induce cells to synthesize the VSV G proteins of the Indiana and New Jersey serotypes, as well as their mutants and chimeras, in quantities sufficient for biological and antigenic studies, we cloned the G genes into plasmid pTF7 used for transient expression in the system recently developed by Fuerst *et al.* (1986). In this system, cultured cells are infected with a recombinant vaccinia virus that expresses the T7 bacteriophage RNA polymerase, followed by transfection with a plasmid containing the gene of interest flanked by the T7 polymerase promoter (ϕ10) and terminator (Tϕ) sequences. The construction and the designation of all plasmids used for expression of VSV glycoproteins, as well as their deletion mutants and chimeras, are summarized in Table 1. Figure 1 shows the restriction maps and the inserts in pWK1 (wt Indiana coding sequence; Fig. 1A) and pWK8 (wt New Jersey coding sequence; Fig. 1B) depicting sites for cleavage by restriction enzymes used to construct all the other plasmids and stick models of the corresponding expression products in relation to their coding sequences. The general features of these G gene expression plasmids are shown in Fig. 1C. Figure 2 presents stick models of all the G protein expression products.

Expression of VSV glycoproteins by pWK plasmids or by vWK vaccinia recombinants

Transfecting plasmids pWK1–pWK16 and infecting vaccinia recombinants vWK1–vWK16, individually co-infected with the vaccinia virus T7 polymerase recombinant vTF1-6,2, were tested for expression of complete, truncated, or chimeric VSV glycoprotein genes (see Table 1). Figure 3 (lanes 1–10) compares the level of expression by four transfecting plasmids with that of four vaccinia virus recombinants carrying the same G gene. The lysates from ~3 × 10^5 cells loaded on the gel contained 50 μg of total cell protein (50–100% of the harvest from a small petri dish or 2–4% of the harvest from a 150-cm^2 flask). Virion proteins (5 μg) of VSV-Ind (Orsay) or VSV-NJ (Ogden) were used as markers. Following electrophoresis, expressed glycoproteins and truncated segments were blotted onto nitrocellulose filters and detected by reaction with a mixture of polyclonal anti-G VSV-NJ and anti-G VSV-Ind rabbit serum followed by ^{125}I-labeled staphylococcal protein A, as described under Materials and Methods.

TABLE 1

DESIGNATION AND ORIGIN OF PLASMID CONSTRUCTS FOR GLYCOPROTEINS EXPRESSED BY INTACT, TRUNCATED, OR CHIMERIC G GENES OF VSV–INDIANA AND VSV–NEW JERSEY CLONED IN pTF7 VECTOR

Plasmid	Cloning vector (enzymes)[a]	Original plasmid (enzyme fragment)[b]	Cloned amino acid sequences[c]		Substitutions and deletions[d]
			VSV–Ind	VSV–NJ	
pWK1	pWK2b(*Stu*I)	pG1(*Stu*I)	1–511	—	
pWK2a	pTF7IHB-1(*Bam*HI)	pTA1(*Xho*I)	IndΔCHO179	—	Thr_{181} → Ala
pWK2b	pTF7IHB-1(*Bam*HI)	pTA2(*Xho*I)	IndΔCHO336	—	Thr_{338} → Ala
pWK2c	pTF7IHB-1(*Bam*HI)	pTA1,2(*Xho*I)	IndΔCHO179/336	—	$Thr_{181,338}$ → Ala
pWK3	pWK1(*Pst*I/*Kpn*I)	(see pWK1)	1–262, 339–511	—	Δ262–338
pWK4	pWK1(*Kpn*I)	(see pWK1)	1–336	—	Gly_{337} → Ala
pWK5	pWK9(*Xho*I/*Bam*HI)	pBSM13G1(*Xho*I/*Xho*II)[e]	1–317	—	318–328
pWK6	pWK1(*Pst*I)	(see pWK1)[f]	1–262	—	263–287
pWK7	pWK2b(*Stu*I)	pG1(*Stu*I)	1–234	—	235–247
pWK8	pWK1(*Xho*I)	pBSM13G2(*Sma*I/*Sph*I)	—	1–517	
pWK9	pWK8(*Xho*I/*Bam*HI)	pBSM13G2(*Hin*P1) pBSM13G1(*Xho*I/*Alu*I)	1–43	43–517	
pWK10	pWK9(*Bam*HI)	pBSM13G1(*Xho*II/*Bam*HI)[e]	1–43, 290–511	43–301	
pWK11	pWK9(*Bam*HI)	pBSM13G1(*Xho*II/*Bam*HI)[g]	1–43, 317–511	43–298	299–320
pWK12	pWK9(*Bam*HI)	pBSM13G1(*Xho*II/*Bam*HI)	1–43	43–298	299–314
pWK13	pWK9(*Bg1*II)	pBSM13G1(*Xho*II/*Bam*HI)	1–43, 317–511	43–267	Δ268–316
pWK14	pWK9(*Xho*I/*Nco*I)	pBSM13G1(*Xho*I/*Nco*I)	1–200	214–517	Δ201–214
pWK15	pWK9(*Xho*I/*Bam*HI)	pBSM13G1(*Xho*I/*Xho*II)[e]	1–297	294–517	
pWK16	pWK14(*Nco*I/*Bg1*II)	(see pWK15)	1–201	321–517	Δ201–320

[a] Plasmids and the restriction enzymes used to prepare the cloning vector.

[b] Restriction enzyme fragments cleaved from the plasmids indicated and cloned into the dephosphorylated cloning vector.

[c] Sequential amino acid sequences of VSV–Ind and VSV–NJ, including the signal sequence.

[d] Amino acid substitutions created by nucleotide point mutations or originating from sequences not in the regular reading frame; amino acid deletions are marked by Δ.

[e] *Xho*II digestions gave rise to incompletely cleaved fragments that were cloned containing one internal *Xho*II site in the case of pWK10 and pWK15 and two internal *Xho*II sites in the case of pWK5.

[f] The *Xba*I/*Bg1*II linker (5′-TCTAGATCTAGA-3′) was inserted in the *Pst*I site of the T4 DNA polymerase-treated vector.

[g] In addition to the *Xho*II fragment representing the carboxy terminus, a small *Xho*II fragment representing amino acids 299–320 has been cloned into the *Bam*HI site opposite to its regular reading frame.

In all cases shown in Fig. 3, co-infection with vTF1-6,2 and the second vaccinia virus recombinant (vWK1, vWK4, vWK9, vWK12) gave higher yields of expressed glycoproteins compared to that of the transient expression by plasmids (pWK1, pWK4, pWK9, pWK12) transfected into cells previously infected with vTF1-6,2. The glycoprotein bands excised from the nitrocellulose were also subjected to scintillation counting and compared with the counts obtained from the glycoprotein bands derived from 5 μg of VSV-Ind or VSV-NJ virions. Based on studies by Thomas *et al.* (1985), a 4- to 15-fold increase in the level of expression was observed when cells were doubly infected with vaccinia viruses bearing G genes (see Fig. 3). The expressed glycoproteins and their truncated forms represented 1.2–1.6% of the total proteins synthesized in the doubly infected cells (data not shown).

Figure 3 also demonstrates that the expression products of pWK1/vWK1 and pWK9/vWK9 exhibit the same electrophoretic migration as the virion glycoproteins, whereas pWK4/vWK4 and pWK12/vWK12 gave rise to the expected truncated 37- and 35-kDa products. pWK5 showed the expected transient expression of the truncated glycoprotein Ind17-317 (Fig. 3, lane 14). All other remaining G-protein-expressing vaccinia virus recombinants were tested with co-infecting vTF1-6,2 for their ability to express the glycoproteins of the anticipated size detectable with polyclonal antiserum (Fig. 3; lanes 11–13 and 15–23). These studies show that all constructs expressed glycoproteins of approximately the size predicted and in fairly good yields with only one exception. No glycoprotein band could be detected by transient expression with pWK8, and recombinant vaccinia virus vWK8 yielded only minute amounts of the expected expression product (data not shown). The deficient expression of pWK8/vWK8 is probably due to the rather unnatural flanking sequences of the ATG start codon in these constructs, which were derived from plasmid pNJG6. Due to the cloning procedure (dG/dC tailing) used to obtain this

type VSV-NJ to individual high-titered homotypic MAbs directed to glycoprotein epitopes V, VI, VII, or VIII to select neutralization-resistant antigenic variants (Luo *et al.*, 1988). In our study, amino acid changes were observed only in the center of the VSV-NJ glycoprotein, whereas Vandepol *et al.* (1986) were able to select variants also having amino acid substitutions at the N-terminus and C-terminus of the VSV-Ind glycoprotein.

In order to obtain a more detailed antigenic map and to determine if the previous postulates on the distribution of epitopes on the VSV-Ind and VSV-NJ glycoproteins are correct, we decided to use recombinant DNA techniques to further investigate the antigenic determinants of the two G proteins. Since the influence of glycosylation on the antigenicity of the G protein is also unclear, we have chosen vectors for expression in a glycosylation-competent eucaryotic system. A vaccinia virus-based transient expression system designed by Fuerst *et al.* (1986, 1987), which had previously enabled us to express high yields of VSV M protein and influenza virus M_1 protein (Li *et al.*, 1988; Baylor *et al.*, 1988), was used for expression of the glycoproteins of VSV as well as their deletion mutants and chimeras. The expressed wild-type, mutant, and chimeric glycoproteins have been tested for binding to monoclonal antibodies by either Western blotting or immuno dotblotting.

MATERIALS AND METHODS

Cells and viruses

VSV-Ind (San Juan strain) and VSV-NJ (Ogden strain) grown in BHK-21 cells were isolated and purified as described previously (McSharry and Wagner, 1971). Vaccinia virus (strain WR), recombinant vaccinia virus containing the T7 RNA polymerase gene (vTF1-6,2), and human TK^-143 cells have been described by Fuerst *et al.* (1986) and were kindly provided by Bernard Moss of the National Institutes of Health. Human TK-143 cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 25 μg/ml of 5-bromo-deoxyuridine (BUdR). CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle medium containing 10% FBS.

Plasmids

The vaccinia virus vector pTF7IHB-1 (Fuerst *et al.*, 1986) was also provided by Bernard Moss. Plasmids pTA1, pTA2, and pTA1,2 containing the entire VSV-Ind G gene with point mutations, which express VSV-Ind G proteins lacking either one (pTA1, pTA2) or both (pTA1,2) carbohydrate chains, were kindly provided by Carolyn E. Machamer (Machamer *et al.*, [1985). Plasmids pG1 (Rose and Gallione, 1981) and pNJG6 (Gallione and Rose, 1983) containing the entire coding sequences of the VSV-Ind and VSV-NJ glycoproteins, respectively, were generous gifts of John K. Rose.

Construction of recombinant plasmids

Restriction and other DNA modifying enzymes were purchased from Bethesda Research Laboratories, Boehringer-Mannheim, or New England Biolabs and were used as directed by the manufacturer. Standard methods (Maniatis *et al.*, 1982) were used for the construction, amplification, and purification of plasmids containing the VSV glycoprotein gene inserts as well as deletion mutants and chimeras. Each of the mutants and chimeras constructed was checked by restriction enzyme mapping (Maniatis *et al.*, 1982) and/or sequencing (Chen and Seeburg, 1985) and by Western blotting for the correct size of its expression product as described below.

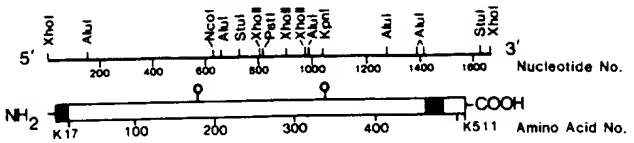
Isolation of recombinant virus

CV-1 cells were infected with wild-type vaccinia virus at a multiplicity of infection (m.o.i.) of 0.05 PFU/cell and transfected with calcium phosphate-precipitated plasmids; 20 to 40 μg of plasmid recombinant DNA was added in 1 ml of HEPES-buffered saline and precipitated by addition of $CaCl_2$ to a final concentration of 125 m*M*. Recombinant viruses formed by insertion of the foreign G gene into the thymidine kinase locus were selected by plaque assay on TK^-143 cell monolayers in the presence of BUdR (25 μg/ml). TK^- recombinant virus plaques were distinguished from spontaneous TK^- mutant virus by DNA–RNA dotblot hybridization. After two consecutive plaque purifications, recombinant virus was amplified by infecting TK^-143 cell monolayers in the presence of BUdR, and then large stocks were made in HeLa cells without selective medium.

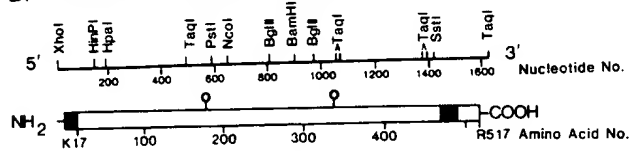
Transient expression of VSV glycoproteins

CV-1 cells were grown to 80% confluence in 35-mm plates (3×10^5 cells) and infected at an m.o.i. of 30 with purified recombinant vaccinia virus vTF1-6,2 expressing T7 RNA polymerase, which is identical to the vTF7-3 recombinant vaccinia virus described by Fuerst *et al.* (1986). The virus was allowed to adsorb for 2 hr at 37° with occasional rocking of the plate. The residual inoculum was then removed, and each plate was washed once with 2 ml of serum-free medium. We then added 0.4 ml of calcium phosphate-precipitated DNA (10 to 30 μg of recombinant plasmid) and, after 15 min, 3 ml of MEM with 5% FBS. After 18 hr of incubation at 37°, the medium was discarded and replaced by fresh MEM with 5% FBS and the incubation was continued at 37°.

A. VSV-Indiana G cDNA and Protein



B. VSV-New Jersey G cDNA and Protein



C. VSV G Protein Expression Vectors

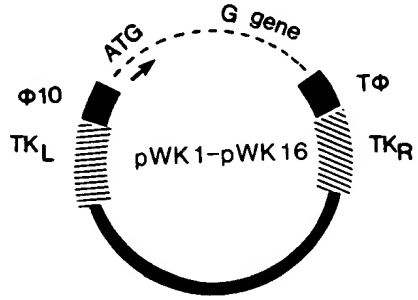


FIG 1. Construction of VSV G gene recombinant plasmids pWK1–pWK16 for creating vectors that express the entire G gene, deletion mutants, truncated genes, and chimeras of VSV-Ind and VSV-NJ G proteins. (A) Restriction enzyme sites in the cDNA of *wt* VSV-Ind G gene and inserts for pWK1, pBSM13G1 and, except for the missing *Kpn*I site, in mutants pTA2, pWK2b, pTA1,2, and pWK2c. Corresponding regions of the G protein are also shown. (B) Restrictions enzyme sites present in the cDNA of the *wt* VSV-NJ G gene of pWK8, and corresponding sequences of the VSV-NJ G protein. The map for plasmid pNJG6 is identical except that the *Xho*I site and *Taq*I site at the ends are replaced by *Pst*I sites. Also identical, except for the missing *Hin*P1 site, is the map of the chimeric insert in pWK9. The hydrophobic N-terminal signal sequence and C-terminal anchor sequences are indicated by black boxes. The two carbohydrate chains are indicated as circles on top of the stick models. Also marked are the N-terminal lysine (K17) and C-terminal lysine (K511 and arginine (R517) amino acids of the mature glycoproteins. (C) Location of the glycoprotein genes in the pWK expression vectors. ϕ10, T7 polymerase promoter; Tϕ, T7 polymerase terminator; TK_L and TK_R, thymidine kinase flanking sequences.

clone, the ATG start codon is preceded by the sequence $G_{15}T$ (Gallione and Rose, 1983). In contrast to vWK8, vWK9 readily expresses the chimeric glycoprotein NJ43-517 upon co-infection with vTF1-6,2 (Fig. 3, lane 7). Since this expressed chimeric glycoprotein contains 95% of the New Jersey glycoprotein sequence and the first 26 VSV-Ind G amino acids are not believed to play a role in the antigenicity of that protein, the expression products of pWK9 or vWK9 can be assumed in subsequent studies to be representative of the wild-type VSV-NJ glycoprotein. Moreover, pWK9

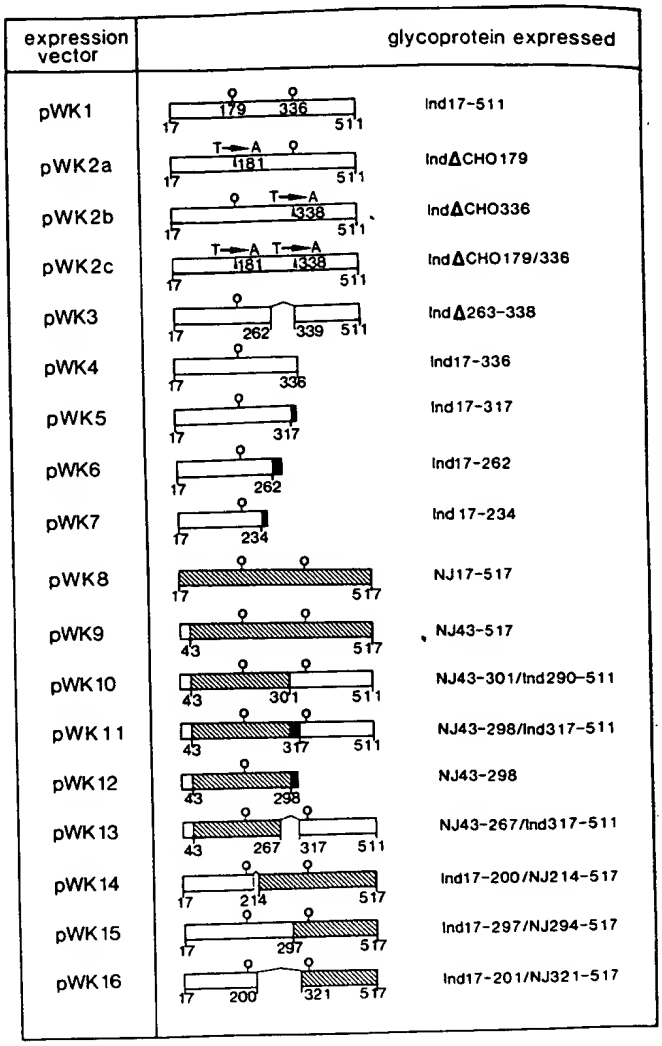


FIG. 2. Stick models of the intact, truncated, and chimeric glycoproteins expressed by plasmids pWK1–pWK16, showing VSV-Ind G protein (open boxes), VSV-NJ G protein (hatched boxes), and amino acid substitutions translated from nucleotide sequences not in the original reading frame (black boxes). The amino acid sequences of the glycoproteins expressed by each corresponding plasmid are displayed as the fully processed G protein after cleavage of the N-terminal signal sequence. The location of carbohydrate chains is shown as circles above the stick models Base changes T → A at nucleotides 1181 and 1338, resulting in the loss of N-glycosylation sites at asparagine residues 179 and/or 336, are shown in the expression products of pWK2a, pWK2b, and pWK2c called IndΔCHO179, IndΔCHO336, and IndΔCHO179/336, respectively. The amino acid sequences expressed by chimeras are indicated by Ind/NJ or NJ/Ind numbers. Despite the overlap of NJ/Ind amino acid sequences, the expression product of pWK10 is a single chimeric protein with VSV-Ind amino acids 290–297 substituting for the homologous VSV-NJ amino acids 294–301 in the expression product NJ43-301/Ind290-511. For the same reason, the expression product of pWK15 was designated Ind17-297/NJ294-517.

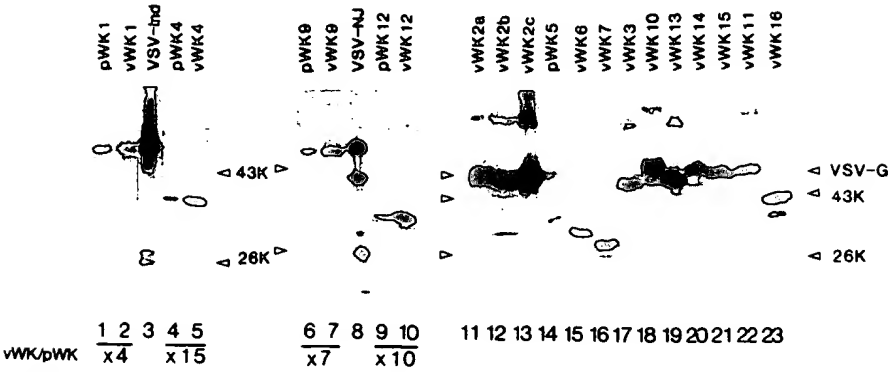


FIG 3. Western blot analysis of glycoproteins expressed in CV-1 cells transfected with plasmids pWK1, pWK4, pWK9, and pWK12 or, for comparison, infected with corresponding vaccinia virus vWK recombinants expressing the same constructs or other constructs driven by the co-infecting vaccinia virus recombinant vTF1-6,2 expressing phage T7 polymerase. Cell extracts containing 50 μg of total protein were loaded on 12.5% polyacrylamide–SDS gels and run under reducing conditions. The separated proteins were transferred to nitrocellulose paper and reacted with a mixture of polyclonal rabbit anti-G VSV-Ind and VSV-NJ sera. Five micrograms of VSV-Ind (lane 3) and 5 μg of VSV-NJ (lane 8) virus were run as marker controls. The positions of 43K and 26K molecular weight markers are also indicated. The glycoprotein bands from lanes 1–10 were excised and subjected to liquid scintillation counting. The increase in the level of G protein expressed by the vWK vaccinia virus vectors compared with that of transfection with the corresponding pWK plasmids is indicated as the ratio of vWK/pWK.

has been used to construct pWK10/vWK10, pWK11/vWK11, pWK12/wVK12, and pWK13/vWK13 which all readily express the appropriate truncated or chimeric glycoproteins as shown in Fig. 3.

Some of the more slowly migrating bands seen in Fig. 3 appear to be dimers or trimers of G proteins and some aberrant, more rapidly migrating bands may be degradation products or prematurely terminated expression products. It is not possible to rule out in certain cases underglycosylated and/or Gs protein lacking the transmembrane carboxy-terminal anchor. Similar minor bands were found by gel electrophoresis after expression of similar constructs (Doms *et al.*, 1988; Machamer and Rose, 1988). The formation of SDS-resistant trimers and other aggregates with varying degrees of reactivity with monoclonal antibodies can also result from mutations in the ectodomain of G protein, including defectiveness or aberrancy of oligosaccharide chains (Machamer and Rose, 1988; Doms *et al.*, 1988).

Mapping G-protein epitopes of VSV-Ind and VSV-NJ by the reactivity of vector-expressed products with monoclonal antibodies

The aims of these experiments were to map antigenic determinants of VSV-Ind and VSV-NJ G proteins by the capacity of the vector-expressed G gene products to bind epitope-specific monoclonal antibodies originally described by Volk *et al.* (1982) and by Bricker *et al.* (1987). The most logical technique for such experiments would appear to be Western blot analysis of pWK1–pWK16 and particularly vWK1–vWK16 expression products in CV-1 cells separated on polyacrylamide gels. As described by Bricker *et al.* (1987) for VSV-NJ MAbs, their binding affinity is often dependent on the secondary structure of the glycoprotein antigen. Moreover, we found that few of the vector-expressed G-gene products would enter the polyacrylamide gel efficiently under non-reducing conditions and often would not enter at all. Therefore, we were only able to use Western blot polyacrylamide gel electrophoresis under reducing conditions for detecting the binding affinity of those few monoclonal antibodies that are not dependent for reactivity on secondary structure of G protein expression products. The majority of antigenic determinants could be detected only by immuno dot-blotting under non-reducing conditions. Chimeric and certain truncated G gene constructs were used to investigate binding affinities of non-cross-reacting MAbs and other truncated constructs were found to be suitable for analyzing the antigenic determinants shared by VSV-Ind and VSV-NJ G proteins as originally noted by Bricker *et al.* (1987). Those plasmids, in which one or both glycosylation sites have been deleted, were also used for screening antigenic reactivity of MAbs dependent on tertiary structure.

(i) Antigenic determinants on VSV-Ind G proteins expressed by glycosylation-defective plasmids. We investigated the influence of glycosylation on the antigenicity of the VSV-Ind G protein by expression of one plasmid and two recombinant vaccinia virus constructs with carbohydrate chain deletions. Plasmid pWK2b was used for transient expression to obtain a VSV-Ind glycoprotein with a Thr → Ala substitution at position 338 lacking the carbohydrate chain at Asn336 (IndΔCHO336). Recombinant vaccinia viruses vWK2a

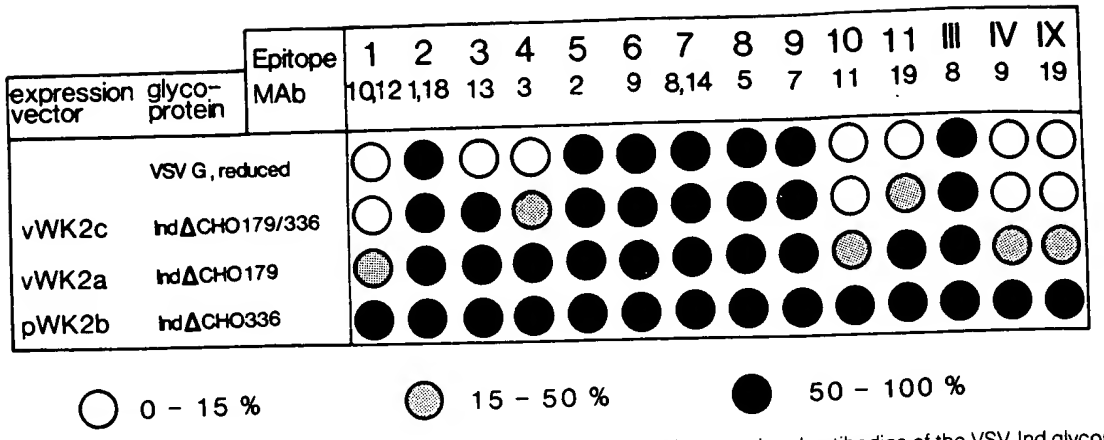


FIG. 4. The influence of secondary structure and glycosylation on the reactivity with monoclonal antibodies of the VSV-Ind glycoprotein either extracted from whole wt virions or expressed by glycosylation-deficient recombinant vectors vWK2c, vWK2a, or pWK2b. In the case of wt virions, equal amounts (5 μg) of VSV-Ind were subjected to electrophoresis in 12.5% polyacrylamide–SDS gels under reducing or nonreducing conditions and transferred to nitrocellulose paper and the G proteins analyzed by Western blotting with MAbs to all nine VSV-Ind G epitopes and three VSV-NJ G cross-reacting epitopes III, IV, and IX. The glycoprotein bands were excised from the nitrocellulose and subjected to liquid scintillation counting. Binding of each MAb to the reduced glycoprotein is expressed as a percentage of binding to the corresponding unreduced glycoprotein. The expression products of vWK1, vWK2a, vWK2c, and pWK2b were obtained as described under Materials and Methods and analyzed by immuno dotblotting with monoclonal antibodies and binding of ^{125}I-labeled staphylococcal protein A followed by liquid scintillation counting. Aliquots of CV-1 cell lysates used in these studies contained 20 μg (vWK1), 30 μg (vWK2a, vWK2c) and 70 μg (pWK2b) of total protein, yielding the same numbers of scintillation counts when reacted with rabbit polyclonal VSV antiserum. Lysates of CV-I cells infected only with vTF1-6,2 and containing the same amount of total protein were used as background controls. Binding of each MAb to the expression products of vWK2a, vWK2c, and pWK2b is displayed as a percentage of its binding to the *wt* glycoprotein expressed by vWK1.

and vWK2c were used for co-infection of CV-1 cells together with vTF1-6,2 to express glycoproteins lacking the carbohydrate chains at Asn179 (IndΔCHO179) and at both glycosylation sites, Asn179 and Asn336, (IndΔCHO179/336), respectively. Lysates of CV-1 cells containing the expressed glycoprotein deletion mutants and the wild-type VSV-Ind G protein were tested by immuno dotblot analysis for their ability to react with VSV-Ind G MAbs and the cross-reactive VSV-NJ G MAbs.

Figure 4 shows that loss of the carbohydrate chain at Asn336 (pWK2b) seems to have little or no influence on the antigenicity of the VSV glycoprotein. If both carbohydrate chains are removed (vWK2c), monoclonal antibodies directed to VSV-Ind G epitopes 1 and 10 and VSV-NJ G epitopes IV and IX recognize the VSV-Ind glycoprotein very poorly or not at all. The same epitopes show reduced reactivity (15–50%) if only the carbohydrate chain at Asn179 is missing. Reduced reactivity (15–50%) for VSV-Ind G epitopes 3 and 11 is also observed if both carbohydrate chains are missing. All other MAbs tested react quite well if only one carbohydrate chain is removed; most of them show somewhat reduced reactivity (50–70%) if the G protein is not glycosylated at all (data not shown).

(ii) Mapping linear epitopes by Western blotting under reducing conditions. MAbs of Indiana epitopes 2, 5, 6, 7, 8, and 9 (see Fig. 4) and of New Jersey epitopes I, II, and III (Bricker *et al.*, 1987) were found to bind their respective G proteins under reducing conditions and, hence, are presumably linear epitopes not dependent on secondary structure. Therefore, these monoclonal antibodies could be tested for their reactivity with vector expression products by Western blotting under reducing conditions. Recombinant vaccinia viruses vWK3, vWK4, vWK10, vWK11, vWK12, vWK13, vWK14, vWK15, and plasmid pWK5 were therefore used to express VSV glycoprotein chimeras and deletion mutants as described under Materials and Methods. The expression products were subjected to electrophoresis on 12.5% polyacrylamide–SDS gels and electroblotted onto nitrocellulose paper. Monoclonal antibodies directed to epitopes 2, 5, 6, 7, 8, and 9 of the VSV-Ind serotype and I, II, and III of the VSV-NJ serotype were tested by Western blot analyses for their capacity to recognize antigenic determinants on the altered G protein products.

Figure 5 shows the results of Western blot analyses performed under conditions by which G proteins are reduced. As noted in Figs. 5A–5B, VSV-Ind epitope-2 MAb16 and epitope-5 MAb2 failed to bind the expression products of vWK4 (Ind17-336) but readily bound to the expression product of vWK3 (IndΔ263-338), indicating that epitopes 2 and 5 are probably located on VSV-Ind G protein carboxy distal to amino acid 338. Supporting evidence for this location of epitopes 2 and 5 comes from the finding that MAb16 (epitope 2), which does not crossreact with VSV-NJ G protein, and

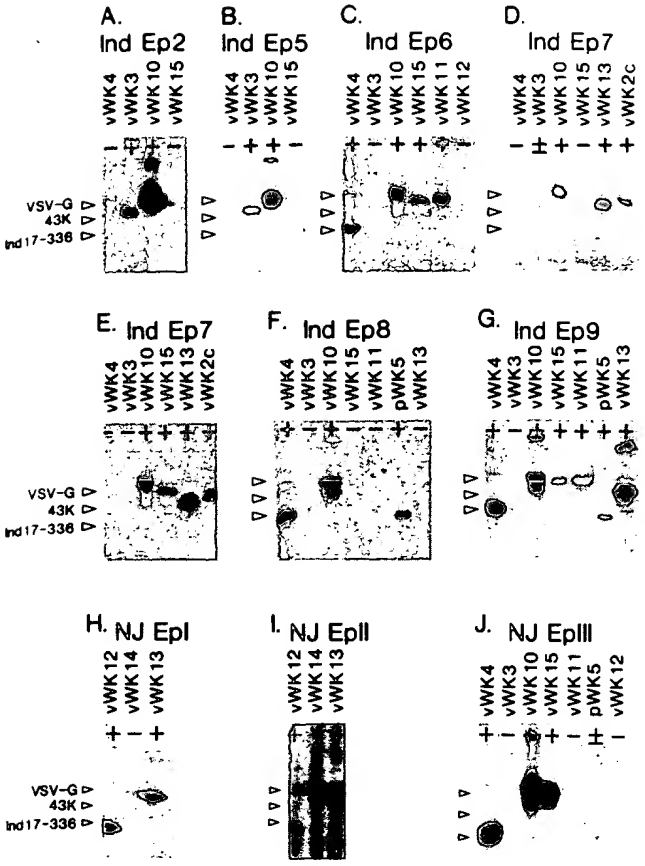


FIG. 5. Western blot analyses of VSV-Ind and VSV-NJ glycoproteins expressed in CV-1 cells transfected with plasmid pWK5 or co-infected with vWK and vTF1-6,2 vaccinia virus recombinants. Cell extracts containing 50 μg of total protein were loaded on 12.5% polyacrylamide slab gels and subjected to electrophoresis under reducing conditions. The separated proteins were then transferred to nitrocellulose paper by electroblotting and then reacted with epitope-specific monoclonal antibodies followed by ^{125}I-labeled staphylococcal protein A. Arrows denote the positions of VSV virion G protein, a 43K protein marker and the vWK4 expression product Ind17-336. The symbols + or − at the top of each lane indicate the reactivity or lack of reactivity of each MAb with the putative expression product. The reactivity of VSV-Ind or VSV-NJ G protein expression products for each MAb is shown in the following panels: (A) MAb16(VSV-Ind epitope 2); (B) MAb2(Ind Ep5); (C) MAb9(Ind Ep6); (D) MAb8(Ind Ep7); (E) MAb14(Ind Ep7); (F) MAb5(Ind Ep8); (G) MAb7(Ind Ep9); (H) MAb2(NJ EpI); (I) MAb5(NJ EpII); and (J) MAb7(NJ EpIII).

MAb2 (epitope 5) both bind exceedingly well to the vWK10 chimeric product NJ43-301/Ind290-511, but fail to recognize the expression product Ind17-297/NJ294-517 of the chimera vWK15 (see Figs. 5A–5B). In a further experiment, it was found that MAbs of VSV-Ind epitopes 2 and 5 bind strongly to a truncated VSV-Ind G protein (Ind8-428) obtained after IPTG induction of *Escherichia coli* cells which had been transfected with plasmid pBSM13+ containing *Bal*31 truncated G gene under control of a *lac* promoter (data not shown). Collectively, these data indicate that epitopes 2 and 5 map to a location in the VSV-Ind G protein between amino acids 339 and 428.

VSV-Ind monoclonal antibodies MAb9 (epitope 6), MAb5 (epitope 8), and MAb7 (epitope 9) can all be mapped to the N-terminal region comprising the first 262 amino acids of the VSV-Ind G protein since they all bind to the truncated protein Ind17-336 expressed by vWK4, but these MAbs do not bind to the deletion mutant IndΔ263-338 expressed by vWK3 (Figs. 5C, 5F, 5G). Due to their cross-reactivity with VSV-NJ G protein, MAb9 (epitope 6) and MAb7 (epitope 9) bind to protein NJ43-301/Ind290-511 expressed by chimera vWK10 and to protein Ind17-297/NJ294-517 expressed by chimera v WK15 (Figs. 5C, 5G), whereas the non-cross-reacting MAb5 (epitope 8) only binds to protein NJ43-301/Ind291-511 expressed by chimera vWK10 (Fig. 5F). These data suggest that epitope-8 MAb5 binds to VSV-Ind G protein in a region between amino acids 290 and 336. Further studies with MAb5 reveal binding affinity to the expression product Ind17-317 expressed by pWK5 but not to the expression product NJ43-267/Ind317-511 of chimera vWK13 or expression product NJ43-298/Ind317-511 of chimera vWK11 (Fig. 5F). These data map epitope 8 of VSV-Ind G protein to a region between amino acid residues 290 and 317. Since MAb5 does not cross-react with the VSV-NJ G protein, its affinity for binding to VSV-Ind G protein must be due to one or more of the nonconserved amino acids 298, 302, 308, 309, 311, and/or 314 in this otherwise highly conserved region. VSV-Ind MAb9 (epitope 6) binds to NJ43-298/Ind317-511 expressed by vWK11 and to NJ43-267/Ind317-511 expressed by vWK13 (data not shown), but it does not react with NJ43-298 expressed by vWK12 although it cross-reacts with VSV-NJ G protein. Therefore, VSV-Ind MAb9 apparently recognizes a region of the VSV-Ind G protein between amino acids 317 and 336.

VSV-Ind MAb7 (directed to epitope 9) cross-reacts with VSV-NJ G protein but does not bind to the expression product NJ43-298 of a truncated VSV-NJ vector (data not shown). However, the same MAb7 binds to the vWK11 expression product NJ43-298/Ind317-511 and to the WK13 expression product NJ43-267/Ind317-511, as well as binding to the truncated protein Ind17-336 expressed by vWK4 and Ind17-317 expressed by pWK5 (Fig. 5G). Therefore, the most reasonable conclusion would seem to be that epitope 9 of VSV-Ind G protein maps to an amino acid sequence spanning both sides of residue 317.

Mapping epitope 7 on VSV-Ind G protein has raised some difficult questions partly because two partially cross-reactive monoclonal antibodies, MAb8 (Fig. 5D) and MAb14 (Fig. 5E), exhibit somewhat different pat-

terns of reactivity with products of certain expression vectors. MAb8 appears to be quite similar to epitope-2 MAb16 (Fig. 5A) and to epitope-5 MAb2 (Fig. 5B) in its pattern of binding to products of the same expression vectors, except for very weak binding to the product IndΔ263-338 expressed by vWK3. On the basis of these results with MAb8, it is tempting to assign a map location of epitope 7 on VSV-Ind G protein to a region carboxy distal to amino acid residue 338. In contrast to MAb8, MAb14 cross-reacts with VSV-NJ G protein and recognizes the vWK15 chimera product Ind17-297/NJ294-517 (Fig. 5E), but in other respects shows a similar pattern of reactivity, or lack of reactivity, with products expressed by vWK4, vWK10, vWK13, and vWK2c. The fact that MAb8 and MAb14 both react strongly with protein NJ43-267/Ind317-511 expressed by the chimera vWK13 suggests a binding site for both MAbs on VSV-Ind G protein distal to residue 317. Competitive binding studies with MAb8 and MAb14, locating them in the same epitope (Volk *et al.*, 1982), suggests overlapping or closely proximal binding sites on VSV-Ind G protein for those two MAbs. Both MAb8 and MAb14 apparently recognize amino acid sequences distal to residue 336 on the basis of failure to bind to the protein Ind17-336 expressed by deletion mutant vWK4. The absence of binding by MAb14 and the weak binding of MAb8 to protein IndΔ263-338 expressed by deletion mutant vWK3 provide a reasonable basis for location of epitope 7 in this deleted region. Although MAb8 may recognize a sequence somewhat distal to amino acid 338, it seems likely that cross-reactive MAb14 binds to a region in the vicinity of amino acids 332–337 because this sequence is conserved in the G proteins of both serotypes VSV-Ind and VSV-NJ (Gallione and Rose, 1983). Both MAb8 and MAb14 bind to the product IndΔCHO179/336 expressed by the glycosylation-deficient mutant vWK2c, although this deleted carbohydrate chain is presumably close to the site for binding of these epitope-7 MAs.

Among the monoclonal antibodies raised against VSV-NJ G protein, those assigned to epitopes I, II, and III react with VSV-NJ G protein subjected to polyacrylamide gel electrophoresis under reducing conditions (Bricker *et al.*, 1987). As shown in Fig. 5H, MAb2 directed to VSV-NJ epitope I binds to the truncated glycoprotein NJ43-298 expressed by vWK12 as well as to the glycoprotein NJ43-301/Ind290-511 expressed by the chimera vWK10 (data not shown). MAb2 (VSV-NJ epitope I) also reacted strongly with glycoprotein NJ43-267/Ind317-511 expressed by chimera vWK13 but did not bind to glycoproteins Ind17-297/NJ294-517 expressed by chimera vWK15 (data not shown) or by Ind17-201/NJ214-511 expressed by chimera vWK14 (Fig. 5H). These findings suggest that epitope I of VSV-NJ G protein is located amino proximal to amino acid 267 and probably amino proximal to amino acid 214. Some additional information pertaining to this map location was obtained by cloning a segment of the VSV-NJ G gene corresponding to amino acids 193–297 in the prokaryotic plasmid pBSM13+ under control of the *lac* promoter; when this recombinant was expressed in *E. coli* on induction with IPTG, the expression product was recognized by VSV-NJ epitope-I MAb2 (data not shown). Conceivably, therefore, epitope I is located between amino acids 193 and 214 of the VSV-NJ G protein.

Figure 5I shows that MAb5 directed to epitope II of VSV-NJ G protein binds to glycoproteins NJ43-298 expressed by vWK12, NJ43-267/Ind317-511 expressed by chimera vWK13 and Ind17-200/NJ214-517 expressed by chimera vWK14. Since VSV-NJ MAb5 does not cross-react with the VSV-Ind G protein (Bricker *et al.*, 1987), it seems clear that epitope II maps to a region between amino acids 214 and 267 on the VSV-NJ G protein.

Unlike MAbs for epitopes I and II, the MAbs directed to epitope III of VSV-NJ G protein readily cross-react with VSV-Ind G protein (Bricker *et al.*, 1987). As shown in Fig. 5J, MAb7 (NJ epitope III) binds strongly to the truncated glycoprotein Ind17-336 expressed by vWK4 but not to the VSV-Ind glycoprotein IndΔ263-338 expressed by the deletion mutant vWK3. Identical results were obtained with the VSV-NJ epitope-III MAb8 (data not shown). As expected from their cross-reactivity with VSV-Ind G protein, MAb7 and MAb8 both bind to chimeric glycoproteins NJ43-301/Ind290-511 and Ind17-297/NJ294-517 expressed by vWK10 and vWK15, respectively (see Fig. 5J). In a separate experiment it was found that MAb7 and MAb8 bind to the vWK14 expression product Ind17-200/NJ214-517 in which amino acids 201–214 are deleted, but MAb7 and MAb8 do not bind to the glycoprotein NJ43-267/Ind317-511 expressed by the chimera vWK13 in which amino acids 268–320 are deleted (see Fig. 7). The most significant finding in this effort to map VSV-NJ epitope III was clear evidence for no binding to MAb7 (or MAb8) to the expression product of chimera vWK11 (NJ43-298/Ind317-514) or to the expression product of the truncated glycoprotein NJ43-298 synthesized by vWK12 (Fig. 5J). Although their binding is very weak, MAb7 and MAb8 do recognize the truncated VSV-Ind glycoprotein Ind17-317 synthesized by pWK5 (Fig. 5J). The consensus derived from these data is that epitope III of the VSV-NJ G protein maps to a region between amino acids 299 and 317.

(iii) Mapping reduction-sensitive epitopes of VSV-Ind and VSV-NJ G proteins by MAb immuno dotblotting. Preliminary studies had shown that many of the mono-

clonal antibodies directed to the G proteins of both VSV-Ind and VSV-NJ, especially those that neutralize viral infectivity, would not react with glycoproteins or glycoprotein fragments under reducing conditions. These finding precluded Western blotting analysis following polyacrylamide gel electrophoresis with reducing agents such as β-mercaptoethanol and dithiothreitol in the gel buffers. Attempts to subject glycoproteins to polyacrylamide gel electrophoresis under non-reducing conditions were also unsuccessful. Therefore, we were forced to resort to immuno dotblotting to analyze those epitopes susceptible to reducing agents ostensibly because of dependence on disulfide bond secondary structure.

Lysates of CV-1 cells co-infected with T7 polymerase-expressing vTF1-6,2 and one of the vaccinia-G gene recombinants (VWK1, vWK3, vWK4, vWK6, vWK7, vWK9, vWK10, vWK12, vWK13, vWK14, vWK15, or vWK16) were tested for their ability to react with MAbs directed to various G protein epitopes of VSV-Ind or VSV-NJ by immo dotblotting analysis, as described under Materials and Methods. The data on neutralizable epitopes are shown in Fig. 6 and the reactivity of MAbs with nonneutralizable epitopes is shown in Fig. 7. Identical results for immuno dotblot and Western blot analyses were obtained in studies of those epitopes not affected by reducing agents (see Fig. 5). The dotblot data recorded in Fig. 6 and Fig. 7 for reaction of recombinant virus-expressed glycoproteins with each MAb are expressed as little or no binding (0–10%), moderate binding (10–50%), or strong binding (50–150%), using as a 100% binding standard the same concentration of *wt* VSV-Ind or VSV-NJ glycoproteins extracted from cells expressing vectors vWK1 or vWK9.

MAbs directed to VSV-Ind epitopes 1, 4, 10, and 11 were first tested by immuno dotblot analysis for their capacity to bind to truncated glycoproteins Ind17-336 expressed by vWK4, Ind17-262 expressed by vWK6 and Ind17-234 expressed by vWK7 as well as the deletion mutant chimera Ind17-201/NJ321-517 expressed by vWK16. The data shown in Figs. 6 and 7 indicate that epitopes 1, 4, 10, and 11 map to a region within the first 200 N-terminal amino acids of the VSV-Ind G protein.

MAb13 of VSV-Ind G epitope 3 was found to bind the truncated glycoprotein Ind17-336 expressed by vWK4 but did not bind to the expression products of vWK6 (Ind17-262), vWK7 (Ind17-234), or vWK13 (NJ43-267/Ind317-511). These results recorded in Fig. 6 indicate that epitope 3 maps to a region between amino acids 263 and 317 on the VSV-Ind G protein.

VSV-NJ G protein appears to have many more antigenic determinants (epitopes) that depend on secondary structure and are susceptible to reducing agents. MAb9 directed to VSV-NJ G epitope IV crossreacts with VSV-Ind G protein and exhibits the same binding pattern as do the MAbs to epitopes 1 and 11 of VSV-Ind G protein (Figs. 6 and 7), thus justifying the map assignment of epitope IV to the first 200 N-terminal amino acids of VSV-NJ G protein. MAb11 (epitope V) and MAb12 (epitope VI) directed to the VSV-NJ G protein do not cross-react with VSV-Ind G protein but were found to react quite well with chimeric glycoproteins NJ43-301/Ind290-511 expressed by vWK10, NJ43-267/Ind317-511 expressed by vWK13, and Ind17-200/NJ214-517 expressed by vWK14, but did not bind to chimeric glycoproteins Ind17-297/NJ294-517 expressed by vWK15 or Ind17-201/NJ321-517 expressed by vWK16 (Fig. 6). These data indicate that epitopes V and VI map to a region on the VSV-NJ G protein between amino acids 214 and 267.

MAb13 and MAb14 directed to epitope VII of the VSV-NJ G protein do not cross-react with the VSV-Ind G protein; both MAbs were capable of binding to the chimeric glycoprotein NJ43-301/Ind290-511 expressed by vWK10 and to the truncated glycoprotein NJ43-298 expressed by vWK12, but neither MAb would react with the chimeric glycoproteins Ind17-297/NJ 294-517 expressed by vWK15, Ind17-201/NJ 321-517 expressed by vWK16, or NJ43-267/Ind 317-511 expressed by vWK13 (Fig. 6). A complication arises because MAb13 reacted strongly with the chimeric glycoprotein Ind17-200/NJ214-517 expressed by vWK14 whereas MAb14 did not. Therefore, the closest approximation for mapping epitope VII is to a region between amino acids 214 and 298 with an additional area amino proximal to position 214 being required for full expression of the binding site for MAb14.

MAb15 directed to epitope VIII of the VSV-NJ G protein also does not cross-react with VSV-Ind G protein but binds to the chimeric glycoproteins NJ43-301/Ind290-511 expressed by vWK10 and less strongly to NJ43-267/Ind317-511 expressed by vWK13, as well as the truncated vWK12-expressed glycoprotein NJ43-298. However, MAb15 did not bind to the chimeric glycoproteins Ind17-297/NJ 294-517 expressed by vWK15 or Ind17-200/NJ214-517 expressed by vWK14 (Fig. 6). These data tend to locate epitope VIII to a region on the VSV-NJ G protein proximal to amino acid 267 with some requirement for sequences proximal to amino acid 214 as well.

Epitope family IX of the VSV-NJ G protein comprises a diverse group of monoclonal antibodies which show partial, often one-way competitive binding for the antigenic determinants (Bricker *et al.*, 1987). Of three monoclonal antibodies assigned to epitope IX, MAb19 and MAb20 exhibited cross-reactivity with products of constructs expressing truncated VSV-Ind G protein

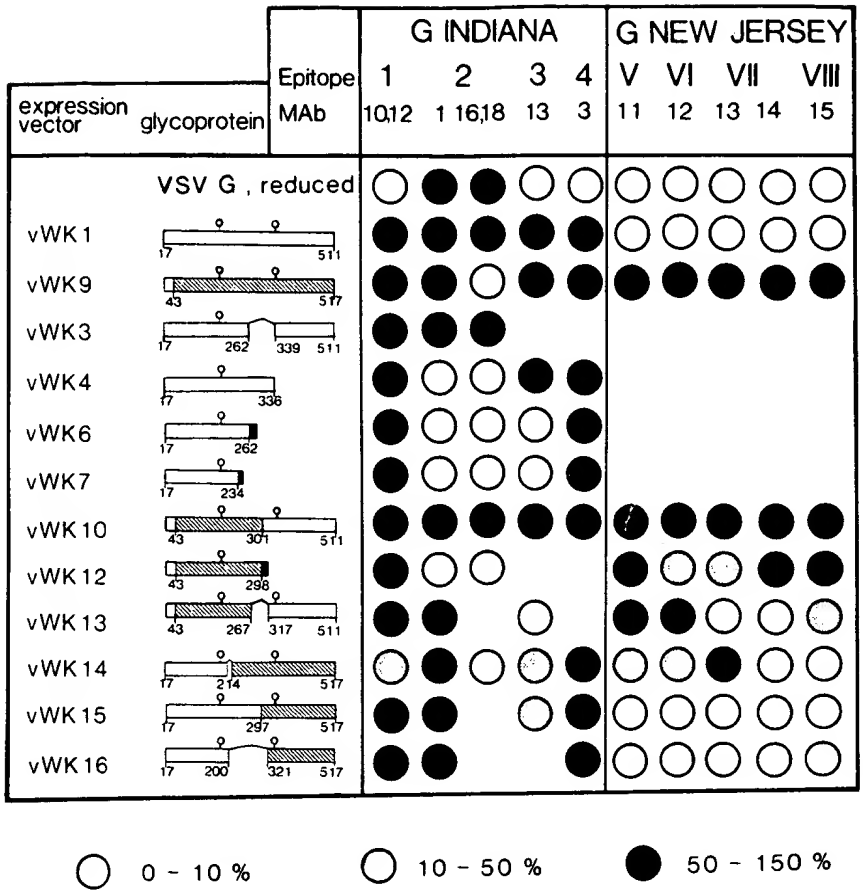


FIG. 6. Binding of epitope-specific neutralizable monoclonal antibodies to VSV-Ind and VSV-NJ glycoprotein expression products as determined by immuno dotblotting under nonreducing conditions. Lysates of CV-1 cells (20–40 μg total protein) that had been coinfected with vWK vectors and vTF1-6,2 contained equal amounts of G protein expression products precipitable by a mixture of polyclonal rabbit VSV-Ind and VSV-NJ anti-G serum. Lysates of cells infected with vTF1-6,2 alone were used as background controls. The data are expressed as three percentage levels of MAb binding (0–10%, 10–50%, and 50–150%), using as the 100% levels the binding of each VSV-Ind MAb to the homotypic *wt* G protein expressed by vWK1 and the binding of each VSV-NJ MAb to the homotypic *wt* G protein expressed by vWK9. Also shown for comparison are results of immuno dotblot binding of these monoclonal antibodies to the disulfide-bond reduced G protein of VSV-Ind virions (also see Fig. 4).

(Ind17-336, Ind17-262, and Ind17-234), whereas MAb22 did not (Fig. 7). However, MAb19 and MAb20 did not react with the glycoprotein Ind17-200/NJ214-517 expressed by the chimera vWK14, thus mapping these binding sites of MAb19 and MAb20 to a region within the first 234 amino acids of the VSV-NJ G protein. Amino acids 201–214, which are deleted in the nonbinding chimeric glycoprotein Ind17-200/NJ214-517, would seem to be a logical region for binding of MAb19 and MAb20; however, the low degree of sequence homology in this region of the VSV-Ind and VSV-NJ G proteins (Gallione and Rose, 1983) makes this an unlikely binding site for MAb19 and MAb20. Consistent with this evidence is the finding that the non-cross-reacting MAb22 of epitope IX did not bind to the chimeric glycoproteins Ind17-297/NJ 294-517 and Ind17-200/NJ214-517 but did bind to the truncated glycoprotein NJ43-298 and to chimeric glycoproteins NJ43-301/Ind290-511 and NJ43-267/Ind317-511 (Fig. 7). These data indicate that epitope IX MAb22 recognizes an area of the VSV-NJ G protein in a region flanked by amino acids 43 and 267.

DISCUSSION

The complex three-dimensional structure of VSV glycoproteins generally precludes mapping antigenic determinants by standard proteolysis techniques. In earlier attempts to map the epitopes of VSV-NJ G protein, we resorted to a "footprinting" method in which monoclonal antibodies coupled to Sepharose beads protected regions of the G protein from digestion by staphylococcal V8 protease. These studies suggested that individual MAbs directed to each of the nine epitopes

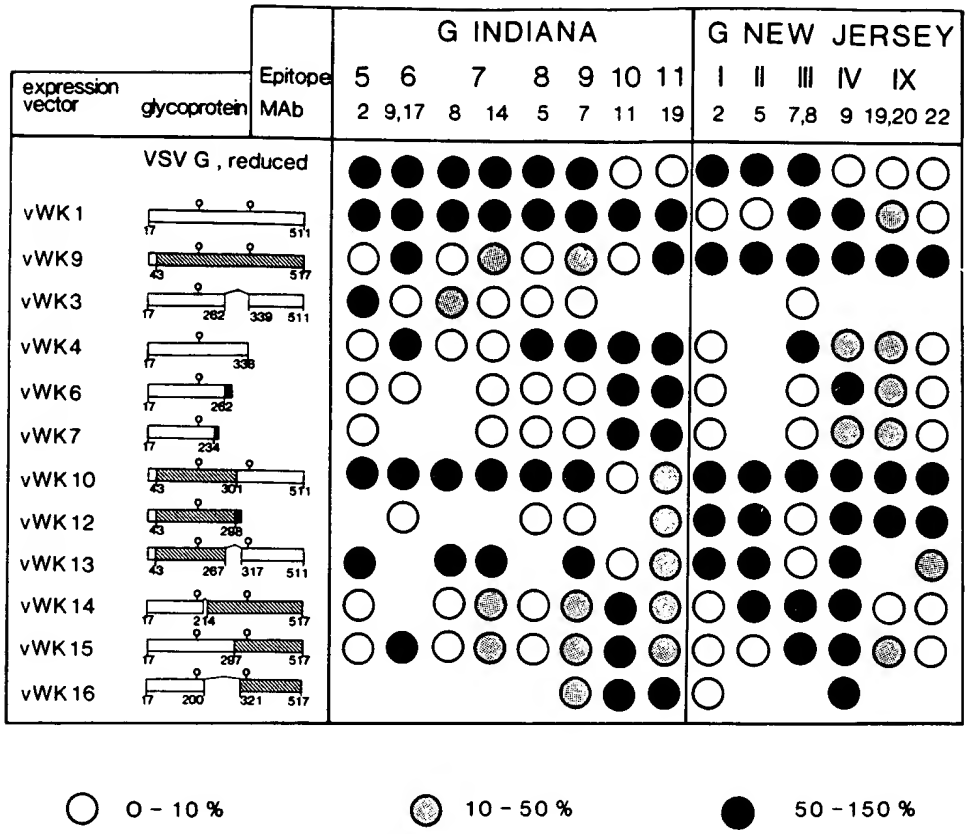


FIG. 7. Binding of epitope-specific nonneutralizable monoclonal antibodies directed to VSV-Ind and VSV-NJ glycoprotein expression products as determined by immuno dotblotting under nonreducing conditions. The experimental procedures are the same as those described in the legend for Fig. 6.

all gave rise to protease-resistant 12-kDa fragments, some of which mapped by N-terminal amino acid sequencing to residues 219–321 of the 517 amino acid G protein of VSV-NJ (Bricker *et al.*, 1987). In other attempts to map the neutralizable G protein epitopes of VSV-Ind (Vandepol *et al.*, 1986) and VSV-NJ (Luo *et al.*, 1988), mutants resistant to neutralization by individual epitope-specific monoclonal antibodies were selected. The virion RNAs of these resistant mutants were then sequenced by primer extension to locate amino acid substitutions in specific regions of the glycoproteins. Table 2 presents a summary of these data on the location of amino acid substitutions in VSV-Ind and VSV-NJ G proteins of neutralizing MAb-resistant mutants compared with the results obtained in the present study on recognition by epitope-specific MAbs of G proteins expressed by deletion mutants and chimeras.

Since the VSV-Ind monoclonal antibodies used by Vandepol *et al.* (1986) are quite different from ours, the sequencing and MAb binding studies for the VSV-Ind G protein cannot be directly compared but probably represent similar epitopes. As noted in Table 2, most, if not all, recombinant-expressed epitopes as well as antibody-induced mutant amino acid substitutions are located in the central third of the VSV-NJ G protein, findings consistent with the footprint mapping data of Bricker *et al.* (1987). In contrast, the VSV-Ind G protein recombinant-expressed epitopes and the neutralization-resistant mutant amino acid substitutions are distributed among the N-terminal, central, and C-terminal thirds of the VSV-Ind G protein. These results suggest that all epitopes on the VSV-NJ G protein are clustered near the center, whereas the epitopes of the VSV-Ind G protein are distributed far more widely. Considering the high degree (50.9%) of amino acid sequence homology between the G proteins of VSV-Ind and VSV-NJ (Gallione and Rose, 1983), one might expect a greater degree of overlap in the locations of the two sets of epitopes. However, there is a considerable degree of antigenic cross-reactivity among the nonneutralizable G protein epitopes of VSV-Ind and VSV-NJ, suggesting selective pressure for diversity of neutralizable epitopes. Quite obviously, one must be cautious in relating epitopes to amino acid substitutions in mutants since allosteric modifications can be exerted by an amino acid substitution at some distance from the

TABLE 2

EPITOPE LOCATIONS ON VSV-IND AND VSV-NJ G PROTEINS MAPPED BY MONOCLONAL ANTIBODIES BINDING TO EXPRESSED RECOMBINANT PRODUCTS COMPARED WITH LOCATIONS OF AMINO ACID SUBSTITUTIONS IN MUTANTS RESISTANT TO NEUTRALIZATION BY EPITOPE-SPECIFIC MONOCLONAL ANTIBODIES

Designation[a]		Amino acid sequences	Mutant substitutions
		A. VSV-Ind G protein epitope map locations	
1	A_2	17–200 (80–183)[b]	Val_{53} → Ile or Ala
4		17–200 (80–183)[b]	Gly_{54} → Ser or Asp
2	B	339–428 (382–400)[b]	Ser_{357} → Pro; Arg_{358} → Ser; Met_{362} → Thr
3	A_1	263–317 (286–317)[b]	Asp_{257} → Tyr or Asn Asp_{259} → Asn; Asp_{263} → Glu
5		339–428	
6		317–336	
7		317–428 (330–350)[c]	
8		290–317 (298–314)[d]	
9		290–336 (310–327)[e]	
10		17–200	
11		17–200 (80–183)[b]	
		B. VSV-NJ G protein epitope map locations	
I		193–267	
II		214–267	
III		299–317	
IV		17–200 (80–183)[b]	
V		214–267	Gly_{364} → Lys
VI		214–267	Pro_{268} → Thr
VII		214–298 (214–289)[d]	Glu_{210} → Lys
VIII		43–267	Ser_{277} → Leu
IX		43–267 (80–228)[b]	

[a] Designations for VSV-Ind G-protein epitopes are consecutive numbers 1–11 based on data of Volk *et al.* (1982) or A_1, A_2, and B based on the data of LeFrancois and Lyles (1982a,b). The order in which these epitopes are listed is arbitrary but based on the comparative locations of the epitopes determined by MAb binding and by amino acid substitutions in VSV-Ind mutants resistant to neutralization by epitope-specific monoclonal antibodies (Vandepol *et al.*, 1986). We tentatively group the neutralizable VSV-Ind epitopes as indicated: 1 and 4, A_2; 2, B; and 3, A_1. The amino acid substitutions in G proteins of VSV-NJ mutants resistant to epitopes V-, VI-, VII-, and VIII-specific MAbs are reproduced from Luo *et al.* (1988).

[b] The parenthetical amino acid sequences indicate more circumscribed areas for locating binding sites of cross-reactive MAbs by excluding amino acid sequences not homologous for VSV-Ind and VSV-NJ G proteins. (Note that VSV-Ind epitope 2 and VSV-NJ epitope IX are capable of binding both cross-reactive and non-cross-reactive MAbs.)

[c] These parenthetical sequences limiting VSV-Ind epitope 7 to a more circumscribed region are based on evidence that epitope 7-specific MAb14 binds to amino acid sequence 317–338, whereas epitope 7-specific MAb8 binds to a region distal to residue 338.

[d] These parenthetical sequences are based on evidence excluding homologous G-protein sequences in both serotypes that do not recognize the non-cross-reactive MAbs of VSV-Ind epitope 8 and VSV-NJ epitope III.

[e] These parenthetical amino acid sequences limiting VSV-Ind epitope 9 to a more circumscribed region on the G protein are based on preliminary data that MAb7 maps to a region in the vicinity of residue 317.

effected epitope, particularly in the case of proteins with complex three-dimensional conformations.

Figure 8 shows linear displays of the comparative map locations reported here for binding of cross-reactive and non-cross-reactive MAbs directed to the expressed products of deletion mutants and chimeras of the VSV-Ind and VSV-NJ G genes. The VSV-Ind epitopes are shown in arabic numerals and the VSV-NJ epitopes in roman numerals. The epitopes involved in virus neutralization are shown in larger type. Some of these epitope map locations are based on lack of sequence homology when considering non-cross-reactive MAbs of both serotypes and excluding nonhomologous regions for cross-reactive MAbs. As noted, VSV-NJ epitopes VIII and IX are aligned along with other epitopes to map location 200–267 even though their MAbs bind definitively only to expression products encompassing amino acids 43–267. MAbs to those two epitopes behaved in a somewhat anomalous manner. For example, the three MAbs of epitope IX did not bind to the chimeric product Ind17-200/NJ214-517 expressed by vWK14, suggesting the contributions of residues 201–214 of VSV-NJ G protein for effective binding of epitope-IX MAbs (see Fig. 7).

Another anomaly is epitope VII of VSV-NJ G protein because MAb14 fails to recognize the chimeric product Ind17-200/NJ214-517, whereas MAb13 directed to the same epitope VII binds quite effectively. These results suggest that amino acid sequences upstream from position 214 contribute to the binding of one of the epitope-VII MAbs but not the other. This interpretation is supported by the data of Luo *et al.* (1988) who reported a Glu → Lys substitution at amino acid 210 in the G protein of a VSV-NJ mutant selected for its resistance to neutralization by the same MAb14 of epitope VII. In addition, the G protein of this MAb14-resistant virus mutant also loses its capacity to bind MAb12 of epitope VI (Luo *et al.*, 1988). Epitope-VI MAb12 does bind to expressed G protein Ind17-200/NJ214-517 (although sequences 201–214 may contribute to binding efficiency), but epitope-VIII MAb15 does not, also suggesting the importance of the amino acid sequence 201–214 (see Fig. 6). In general, the data presented here on mapping the epitopes of VSV-NJ G protein by MAbs binding to expression products of deletion mutants and chimeras are in agreement with the "footprinting" data of Bricker *et al.* (1987). The only significant divergence is epitope IV, which was mapped to the amino acid sequence 17–200, or by cross-reactivity with VSV-Ind G protein constructs, to positions 80–183, rather than sequences downstream from residue 214 reported by Bricker *et al.*, (1981). Although none of the amino acid substitutions in mutants resistant to neutralization by MAbs directed to epitopes VI, VII, and

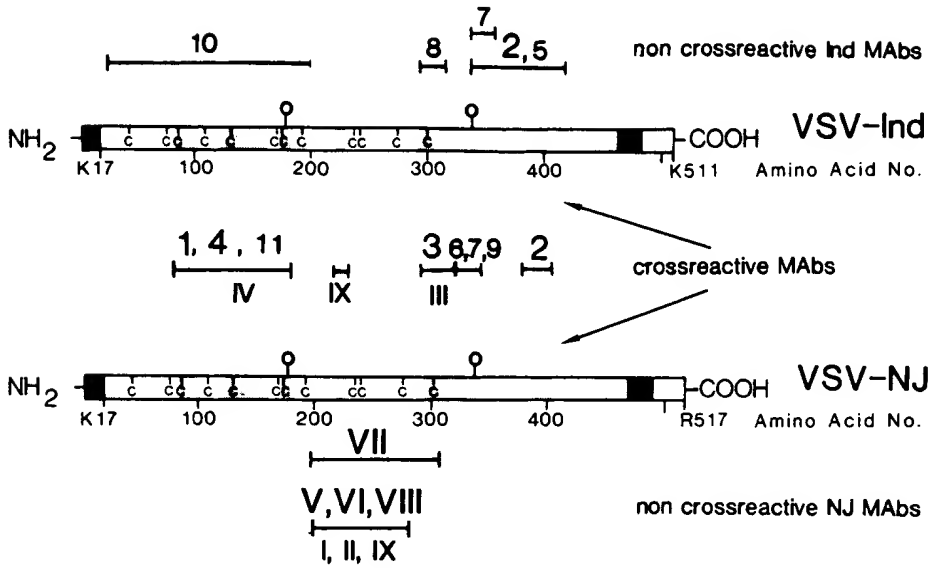


FIG. 8. Summary map showing the postulated locations of the G-protein epitopes of VSV-Ind and VSV-NJ. The epitopes involved in binding the non-cross-reactive monoclonal antibodies to the G proteins are shown above the stick model for VSV-Ind and below the stick model for VSV-NJ. The epitopes involved in the binding of cross-reactive monoclonal antibodies to both VSV-Ind and VSV-NJ G proteins are shown between the two stick models. Arabic numerals designate the G protein epitopes of VSV-Ind and roman numerals are for those of VSV-NJ. The larger numbers denote the epitopes specific for neutralizing monoclonal antibodies. Regions with sequence homology of 75% or greater are shown as stippled areas. Black boxes represent the hydrophobic amino acids of the signal and transmembrane anchor sequences. Carbohydrate chains are indicated by circles above the stick models and the conserved cysteine residues by the letter c inside the boxed areas.

VIII (Luo *et al.*, 1988) occurred directly within the epitope sequences mapped here, all three were extremely close to the relevant MAb binding sites. The major exception was the amino acid substitution Glu → Lys at position 364 in the G protein of the mutant resistant to neutralization by epitope-V MAb11, well outside the sequence 214–267 mapped as epitope V by binding of MAb11 to expressed products of deletion mutants and chimeras of the VSV-NJ G gene. One can only speculate that an amino acid substitution at position 364 can alter the three-dimensional structure of the VSV-NJ G protein to such a degree that it alters the MAb binding capacity of epitope V ostensibly located within the amino acid sequence 214–267.

Figure 8 demonstrates that none of the 11 VSV-Ind epitopes maps to the central region (amino acids 200–285) of the G protein as do all 9 VSV-NJ epitopes except epitopes III and IV, which cross-react with VSV-Ind G protein. One of the two domains for the VSV-Ind G protein epitopes is located within the first 200 N-terminal amino acids, comprising neutralizable epitopes 1 and 4 as well as the nonneutralizable epitopes 10 and 11; a more detailed map location of epitopes 1, 4, and 11 can probably be assigned to a region of the VSV-Ind G protein extending from residues 80 to 183 based on cross-reactivity of these epitope-specific MAbs with partially homologous regions of the VSV-NJ G protein. All VSV-Ind epitopes located in the N-terminal domain, particularly epitopes 1 and 10, depend on an intact glycosylation site at residue 179, based on substantial loss in reactivity with monoclonal antibodies when Asn179 of pWK2a is not glycosylated. This finding suggests that these epitopes may lie in close proximity to the glycosylation site at residue 179 or the carbohydrate chain itself is responsible for the structural integrity required for recognition of the epitopes by the respective MAbs. Moreover, as in the case of the VSV-NJ epitopes, VSV-Ind epitopes 1, 4, 10, and 11 depend on intact secondary structure of the G protein for antigenic activity due, almost undoubtedly, to the presence of 12 cysteine residues, and probably six disulfide bonds, in the region of the first 300 N-terminal amino acids.

The seven remaining VSV-Ind G-protein epitopes are apparently clustered in a carboxydistal domain extending from amino acid residue 286 to 428 (Fig. 8). In contrast to the VSV-Ind epitopes in the cysteine-rich N-terminal domain, none of the carboxy-terminal epitopes depends on secondary structure for their antigenic reactivity except the neutralizable epitope 3, which may depend on the cysteine at position 300 for the secondary structure which determines its antigenic reactivity. Presumably due to the absence of cysteine residues beyond position 300, all the other carboxy-terminal epitopes of VSV-Ind G protein, including the neutralizable epitope 2, react with their respective MAbs equally well

in the reduced or the unreduced state. In contrast to the N-terminal epitopes, the carbohydrate chain attached to the asparagine residue at position 336 does not appear to influence the antigenic specificity of any of the carboxy-terminal epitopes, as demonstrated by retention of these epitopes in the mutant pWK2b. Despite potential steric hindrance by this carbohydrate chain, the region adjacent to Asn336 gives rise to monoclonal antibodies directed to epitopes 6, 7, and 9. In this respect, the VSV-Ind G protein differs from the influenza virus hemagglutin in (Skehel *et al.*, 1984) and the rabies virus glycoprotein (Wunner *et al.*, 1985), both of which lose certain antigenic determinants in mutants bearing additional carbohydrate chains.

In partial conflict with our data, Vandepol *et al.* (1986) located amino acid substitutions in the central, as well as the N-terminal and C-terminal region of VSV-Ind neutralization-resistant mutants. Not unlike our hypothesis for the VSV-NJ G protein, they postulate the existence of an antigenic loop for their major A1 epitope located at the center of the VSV-Ind G protein. As noted above, allosteric influences on the complex three-dimensional structure of such proteins can alter antigenic specificities at sites far distant from mutational alterations. It should also be noted that the amino acid substitutions reported by Vandepol *et al.* (1986) and by Luo *et al.* (1988) in VSV-Ind and VSV-NJ G proteins, respectively, also result in altered charge that could affect conformation leading to altered antigenic reactivity of the two glycoproteins at sites distant from the mutations. Another possible contributing factor to the discrepancy in our results and those of Vandepol *et al.* (1986) could be the fact that we are studying the glycoprotein of the San Juan strain of VSV-Ind, the sequence of which (Rose and Gallione, 1981) differs in 14 amino acids from that of the Mudd-Summers (MSB) strain used by Vandepol *et al.* (1986). Of course, we also used different monoclonal antibodies.

In a summary of the data presented here, based on reactivity of specific monoclonal antibodies with expressed products of deletion mutants and chimeras, the epitopes of the VSV-Ind and VSV-NJ glycoproteins map to different domains despite a considerable number of shared epitopes and 50% amino acid sequence homology between the two glycoproteins. As pointed out by an astute reviewer of this paper, "it is not known whether any of the hybrid proteins or deleted proteins are transported properly" or aberrantly glycosylated; we cannot rule out this possibility but the carbohydrate-deficient expression vectors suggest such antigenic alterations are limited to a few epitopes. Clearly, precise location of antigenic determinants in these two glycoproteins must await detailed crystallographic analysis.

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REFERENCES

BAYLOR, N. W., LI, Y., YE, Z., and WAGNER, R. R. (1988). Transient expression of the matrix (M_1) gene of WSN influenza A virus in a vaccinia vector. *Virology* **163,** 618–621.

BISHOP, D. H. L., REPIK, P., OBIJESKI, J. F., MOORE, N. F., and WAGNER, R. R. (1975). Restitution of infectivity to spikeless vesicular stomatitis virus by solubilized viral components. *J. Virol.* **16,** 75–84.

BRICKER, B. J., SNYDER, R. M., FOX., J. W., VOLK, W. A., and WAGNER, R. R. (1987). Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus (New Jersey serotype): A method for preliminary mapping of epitopes. *Virology* **161,** 533–540.

CARROLL, A. R., and R. R. WAGNER. (1979). Role of the membrane (M) protein in endogenous inhibition of in vitro transcription of vesicular stomatitis virus. *J. Virol.* **29,** 134–142.

CHEN, S. S.-L., DOHERTY, R., O'ROURKE, E. J., ARIEL, N., and HUANG, A. S. (1987). Effects of transport inhibitors on the generation and transport of a soluble viral glycoprotein. *Virology,* **160,** 482–484.

DOMS, R. W., RUUSALA, A., MACHAMER, C., HELENIUS, J., HELENIUS, A., and ROSE, J. K. (1988). Differential effects of mutations in three domains on folding, quaternary structure and intracellular transport of vesicular stomatitis virus G protein. *J. Cell Biol.* **107,** 89–99.

FUERST, T. R., EARLY, P. L., and MOSS, B. (1987). Use of hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.* **7,** 2538–2544.

FUERST, T. R., NILES, E. G., STUDIER, F. W., and MOSS, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83,** 8122–8126.

GALLIONE, C. J., and ROSE, J. K. (1983). Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotypes of vesicular stomatitis virus. *J. Virol.* **46,** 162–169.

KELLEY, J. M., EMERSON, S. U., and WAGNER, R. R. (1972). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *J. Virol.* **10,** 1231–1235.

LEFRANCOIS, L., and LYLES, D. S. (1982a). The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. *Virology* **121,** 157–167.

LEFRANCOIS, L., and LYLES, D. S. (1982b). The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. II. Monoclonal antibodies to non-neutralizing and cross-reactive epitopes. *Virology* **121,** 168–174.

LI, Y., LUO, L., SNYDER, R. M., and WAGNER, R. R. (1988). Expression of the M gene of vesicular stomatitis virus cloned in various vaccinia virus vectors. *J. Virol.* **62,** 776–782.

LINGAPPA, V. R., KATZ, F. N., LODISH, H. F., and BLOBEL, G. (1978). A signal sequence for the insertion of transmembrane glycoprotein. *J. Biol. Chem.* **253,** 8867–8870.

LUO, L., LI, Y., SNYDER, R. M., and WAGNER, R. R. (1988). Point mutations in the glycoprotein gene of vesicular stomatitis virus (New

Jersey serotype) selected by resistance to neutralization by epitope-specific monoclonal antibodies. *Virology* **163,** 341–348.

MACHAMER, C. E., FLORKIEWICZ, R. Z., and ROSE, J. K. (1985). A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis G protein to the surface. *Mol. Cell. Biol.* **5,** 3074–3083.

MACHAMER, C. E., and ROSE, J. K. (1988). Influence of new glycosylation sites on expression of vesicular stomatitis G protein at the plasma membrane. *J. Biol. Chem.* **263,** 5948–5964.

MANIATIS, T., FRITSCH, E. F., and SAMBROOK, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

MATLIN, K., REGGIO, A., SIMONS, K., and HELENIUS, A. (1982). Pathway of vesicular stomatitis virus entry leading to infection. *J. Mol. Biol.* **156,** 609–631.

MCSHARRY, J. J., and WAGNER, R. R. (1971). Lipid composition of purified vesicular stomatitis virus. *J. Virol.* **7,** 59–70.

PAL, R., GRINNELL, B. W., SNYDER, R. M., WIENER, J. R., VOLK, W. A., and WAGNER, R. R. (1985). Monoclonal antibodies to the M protein of vesicular stomatitis virus (Indiana serotype) and to a cDNA M gene expression product. *J. Virol.* **55,** 298–306.

ROSE, J. K., and GALLIONE, C. J. (1981). Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* **39,** 519–528.

SKEHEL, J. J., STEVENS, D. J., DANIELS, R. S., DOUGLAS, A. R., KUOSSOW, M., WILSON, I. A., and WILEY, D. C. (1984). A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **81,** 1779–1783.

THOMAS, D., NEWCOMB, W. W., BROWN, J. C., WALL, J. G., HAINFELD, J. F., TRUS, B. L., and STEVEN, A. C. (1985). Mass and molecular composition of vesicular stomatitis virus: A scanning transmission electron microscopy analysis. *J. Virol.* **54,** 598–607.

TOWBIN, H., STAEHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

VANDEPOL, S. B., LEFRANCOIS, L., and HOLLAND, J. J. (1986). Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus. *Virology* **148,** 312–325.

VOLK, W. A., SNYDER, R. M., BENJAMIN, D. C., and WAGNER, R. R. (1982). Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: Comparative neutralizing activity. *J. Virol.* **42,** 220–227.

WAGNER, R. R. (1987). Rhabdovirus biology and infection: An overview. *In* "The Rhabdoviruses" (R. R. Wagner, Ed.), pp. 9–74. Plenum, New York.

WUNNER, W. H., DIETZSCHOLD, B., SMITH, C. L., LAGON, M., and GOLUB, E. (1985). Antigenic variants of CVS rabies virus with altered glycosylation sites. *Virology* **140,** 1–12.

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Host Range Deletion Mutant of Vaccinia Virus Defective in Human Cel

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A vaccinia virus host range (hr) mutant unable to multiply in most human cell lines assayed has been isolated after nitrous acid mutagenesis. This mutant also displayed various alterations in plaque morphology and cytopathic effect on permissive cell lines. The block in multiplication in human cells was at an early stage of infection. Only early cytoplasmic RNA and early viral-induced polypeptides could be detected and there was no evidence of morphological events within viroplasms. Protein synthesis constantly declined as infection proceeded suggesting either that early viral mRNA was unstable or that the mutant virus was defective in a step necessary for the maintenance of translation. Restriction enzyme digestion of DNA from the hr mutant revealed that it was deleted of about 12.6×10^6 daltons in the left-hand end of the genome leaving intact a fragment containing the terminal crosslink. In both permissive and nonpermissive cells the mutant failed to induce the synthesis of an early 42K polypeptide which could thus be encoded within a region of the deleted sequence. The failure to segregate the various phenotypic and biochemical properties of the hr mutant from one another through recombination with a temperature sensitive mutant indicated that the pleiotropic characteristics of the mutant virus were due to the deletion.

INTRODUCTION

Vaccinia virus has a very broad host range among the mammalian and avian cells that have been tested for its growth. This is partially explained by the considerable autonomy the virus possesses due to the numerous viral functions coded for by its large genome (for a review see Moss, 1974). Vaccinia virus is most likely also endowed with specific genes encoding for information that enables it to cross species barriers. The existence of such genes has been suggested by the isolation of host range mutants of vaccinia virus (Tagaya *et al.*, 1961; Gangemi and Sharp, 1978) and the closely related rabbitpox virus (Gemmell and Fenner, 1960; McClain, 1965; Sambrook *et al.*, 1966; Fenner and Sambrook, 1966). Studies with host range mutants should allow both the identification of the polypeptides required for growth in various cell types, the determination of the function of these polypeptides and the localization of the genes coding for them on the virus genome. The purpose of this report is to describe the isolation and the phenotypic and biochemical characterization of a vaccinia virus host range mutant that exhibits complete defectiveness in multiplication only in certain human cells.

MATERIAL AND METHODS

Cells. The following cell cultures were used in this study: primary chick embryo fibroblasts (CEF), hamster BHK 21, mouse L and DBT cells, monkey primary kidney and CV_1, human KB, Hep 2, NCTC 2544, Detroit 550 (American Type Culture Collection CCL 109), human embryonic fibroblasts line 809 provided by Dr. Boué (Paris), MRC 5 (ATCC, CCL 171), and two human embryo cell lines designated HE1 and HE2 prepared in our laboratory. Cells were grown in monolayers with Eagle's basal medium or modifications of it and supplemented with 10% calf serum.

Virus. The vaccinia virus strain used in

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ıs Defective in Human Cells

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IATERIAL AND METHODS

The following cell cultures were is study: primary chick embryo fi- (CEF), hamster BHK 21, mouse 3T cells, monkey primary kidney human KB, Hep 2, NCTC 2544, 50 (American Type Culture Col- CL 109), human embryonic fibro- e 809 provided by Dr. Boué RC 5 (ATCC, CCL 171), and two ıbryo cell lines designated HE1 prepared in our laboratory. Cells wn in monolayers with Eagle's ium or modifications of it and sup- l with 10% calf serum.

The vaccinia virus strain used in this study was thought to be a Lister strain. However, restriction enzyme digests of viral DNA demonstrated that our strain was more closely related to a number of other vaccinia strains than to Lister (see Results). Research concerning its origin has revealed that it was derived from a Copenhagen strain used for vaccination in Denmark and Holland. Prior to mutant isolation the virus was plaque purified on CEF monolayers. The virus stock obtained was submitted to nitrous acid (0.2 *M*) mutagenesis for 8 min according to Fried (1965). Mutagen treatment reduced the virus titer from 3.5×10^6 to 9.3×10^4. Mutagenized virus was then cloned by endpoint dilution on BHK cells at 29°. Out of 247 clones tested one was found to be temperature sensitive at 39.5° and another was the plaque morphology mutant, subsequently characterized as a host range mutant, described herein. The latter mutant was again plaque purified and stocks were produced on BHK cells.

Vaccinia virus recombinants harboring both the temperature sensitive mutation ts_2 (Drillien *et al.*, 1978a) and the host range mutation were produced by mixedly infecting CEF monolayers with 5 PFU per cell of each mutant and incubating at 33° for 24 hr. Recombinants were selected from the yield of the mixed infection in one of the following manners.

(1) Plaques were formed under an agar overlayer on CEF at 33° for 3 days and their plaque morphology determined. The dishes were then shifted to 39.5° for 2 days. Plaques that exhibited the plaque morphology desired and failed to enlarge were picked. Two of the twelve plaques thus isolated actually bred true.

(2) One hundred plaques were randomly picked from CEF infected monolayers and tested for temperature sensitivity on CEF and host range on the human 809 cell line. Three of them exhibited both host range and temperature sensitivity.

Incorporation of labeled precursors into DNA and RNA. DNA and RNA syntheses were followed by pulse labeling infected or uninfected KB cell monolayers (about 5×10^6cells), respectively, with [^{3}H]thymidine (20 μCi/ml) for 30 min or [^{3}H]uridine (10 μCi/ml) for 10 min at various intervals after infection. At the end of the labeling period cytoplasmic fractions were prepared as previously described (Drillien *et al.*, 1977), precipitated with 10% trichloroacetic acid (TCA), collected on glass fiber filters, and counted.

Protein synthesis and polyacrylamide gel electrophoresis. Approximately 2×10^6 cells were labeled with [^{35}S]methionine (5 μCi/ml) for 2 hr. Cells were then recovered from the dishes and solubilized in 0.1 ml dissociating buffer (Tris–hydrochloride, 0.05 *M*, pH 6.8), sodium dodecyl sulfate (2%), β-mercaptoethanol (2%), glycerol (15%), and bromophenol blue (0.001%).

Polypeptides were analyzed after heating samples at 100° in a boiling water bath for 3 min. Equivalent amounts of labeled material were submitted to gel electrophoresis on 10% polyacrylamide slab gels according to the technique described by Laemmli (1970) and Studier (1973). After electrophoresis, gels were stained with Coomassie brilliant blue, destained, and processed for fluorography (Bonner and Laskey, 1974). The dried gels were exposed to Kodak RP X-Omat film at −80°.

Incorporation into protein was measured after TCA precipitation of aliquots onto Whatman 3 MM filter squares. The filters in 10% TCA were heated in a boiling water bath for 20 min, extensively washed with TCA, with ethanol, dried, and counted.

Purification, restriction enzyme digestion, and agarose gel electrophoresis of viral DNA. The DNA from purified virus particles was extracted according to the procedure of Parkhurst and Heidelberger (1976) modified by McFadden and Dales (1979). DNA was digested with *Hin*dIII (Bio Lab) for 4 hr at 37° in 10 m*M* Tris–HCl (pH 7.4), 50 m*M* NaCl, 10 m*M* $MgCl_2$, 14 m*M* dithiothreitol. The reaction was stopped by adding an 0.5 vol of 50 m*M* EDTA, 10, m*M* Tris–HCl (pH 8), 50% sucrose, 0.125% bromophenol blue. DNA fragments were separated on horizontal slab gels of 0.6% agarose in 40 m*M* Tris–acetate (pH 8), 20 m*M* sodium acetate, 1 m*M* EDTA. Electrophoresis was carried out overnight at 30 V and gels were stained with 1 μg/ml ethidium bromide. Fragments

were visualized with ultraviolet light and photographed. Lambda DNA digested with *Hin*dIII (Boehringer Mannheim) served as a molecular weight marker. Terminal crosslinked fragments (Geshlin and Berns, 1974) were detected after agarose gel electrophoresis of digested DNA that had been submitted to denaturation with 60% (v/v) formamide at 60° for 6 min followed by rapid cooling (Moyer and Rothe, 1980).

RESULTS

Isolation and Phenotypic Characterization

The host range mutant (hr) to be described in this study was originally isolated as a plaque morphology mutant during screening for temperature sensitive mutants in a nitrous acid mutagenized stock. The plaques produced by this mutant were smaller than the average wild-type plaque on BHK, CEF, and CV_1 cells. Moreover they could be distinguished from the occasional small wild-type plaque by the type of cytopathic effect produced. Whereas the wild type aggregated BHK and CEF cells so as to produce areas of clearing within the plaques of the monolayer the hr mutant failed to do so and plaques could only be identified due to reduced staining with neutral red. In CV_1 cells the hr mutant induced extensive polykaryocytosis (each plaque consisted of several multinucleate cells) not obtained with the wild type.

The host range of the mutant was tested on 14 different cell lines (Table 1). The data illustrate that most of the human cell lines tested, epithelial or fibroblastic, transformed (KB) or diploid (809), were nonpermissive since no increase in titer was found after 36 hr of infection. In several cases assayed (KB, 809) no plaques could be detected on hr-infected monolayers even at high m.o.i. The host range phenotype was also found to be independent of the temperature of the experiment. In contrast to the above results two human embryo cell lines (HE1, HE2) were permissive for mutant growth albeit to a very limited extent. The monkey cells that were assayed for hr multiplication (one primary cell culture and one continuous cell line) were less permissive than cells of hamster, mouse, or chick origin and may be considered as semipermissive compared to the latter. The titers obtained with the hr mutant on permissive cells were often slightly lower than wild-type titers this being in agreement with the fact that plaques produced by the mutant were usually smaller than wild-type plaques. The existence of three kinds of infections with the mutant virus either permissive, semipermissive, or nonpermissive is clearly illustrated in comparative growth curves (Fig. 1). Regardless of the m.o.i. the hr mutant always increased to titers about 10-fold lower than the wild type in

TABLE 1

MULTIPLICATION[a] OF VACCINIA VIRUS hr MU AND WILD TYPE IN DIFFERENT CELL LI

Cell type	Virus wt	Virus hr	Per wt
Human			
KB	200	0.5	C
Hep 2	800	0.2	C
$NCTC_{2544}$	454	0.1	C
Detroit 550	428	0.2	C
809	200	0.3	C
HE_1	1230	28	2
He_2	714	24	3
MRC 5	333	2.3	C
Monkey			
Primary kidney	714	25	3
CV_1	850	80	9
Mouse			
L	833	400	48
DBT	100	67	67
Hamster			
BHK	625	300	48
Chick			
CEF	1600	882	55

[a] Cell monolayers were infected at an approx multiplicity of 0.1 PFU per cell for 36 hr at 37°. T values determined for the wild-type and the hr mut represent the ratio of the titers obtained after 33 h of infection over the titer in the cultures at the end o the adsorption period.

[b] The percentage was obtained by dividing the ra tios obtained for the hr mutant by those obtained fo the wild type. ×100.

TABLE 1

CATION[a] OF VACCINIA VIRUS hr MUTANT ILD TYPE IN DIFFERENT CELL LINES

ype	Virus wt	Virus hr	Percentage wt yield[b]
	200	0.5	0.25
	800	0.2	0.03
u	454	0.1	0.02
550	428	0.2	0.05
	200	0.3	0.15
	1230	28	2.3
	714	24	3.4
	333	2.3	0.7
kidney	714	25	3.5
	850	80	9.4
	833	400	48
	100	67	67
	625	300	48
	1600	882	55

onolayers were infected at an approximate of 0.1 PFU per cell for 36 hr at 37°. The rmined for the wild-type and the hr mutant the ratio of the titers obtained after 36 hr over the titer in the cultures at the end of tion period.

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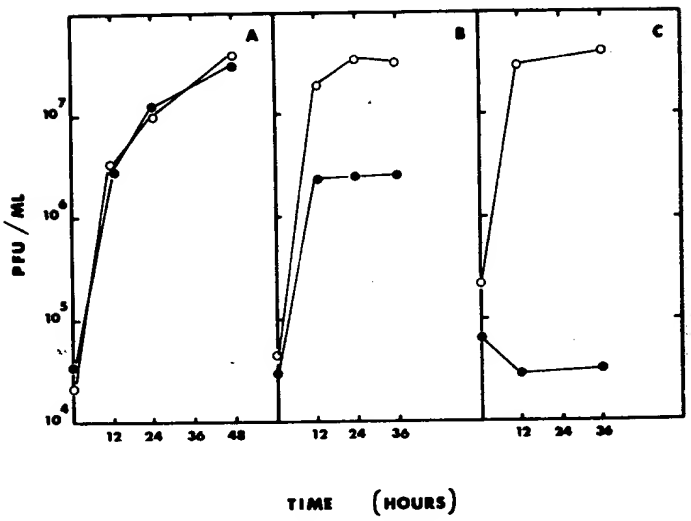


FIG. 1. Multiplication of the hr mutant and the wild type in three different cell types. Cell monolayers were infected either with the wild type (○) or with the hr mutant (●) at a multiplicity of 0.1 PFU per cell and at the indicated times, infection was arrested by freezing the cultures. The titers were subsequently determined by the plaque assay on BHK monolayers. (A) Chick embryo; (B) monkey CV 1 cells; (C) human KB cells.

CV 1 cells and failed to multiply at all in KB cells.

Defectiveness of Early Events in hr-Infected Nonpermissive Cells

The nonpermissive human KB cell line was chosen for analysis of the various stages of vaccinia virus development. Experiments were carried out to determine both the amount of cytoplasmic RNA and DNA syntheses as well as the amount of total protein synthesis after infection with the wild-type or the hr mutant (Fig. 2). Early after infection with the hr mutant (up to 90 min) cytoplasmic RNA synthesis was enhanced relative to mock-infected cells to a similar extent as in the wild-type infection whereas after this period only wild-type-infected cells exhibited a high level of RNA synthesis (Fig. 2a). This indicates that early but not late transcription of the hr genome occurred. Cytoplasmic DNA synthesis was considerably lower in hr than in wild-type-infected cells (Fig. 2b). Thus the very low level of hr DNA synthesis may explain the lack of late RNA. With regard to total protein synthesis there was a progressive decline in amino acid incorporation in hr-infected relative to the wt-infected cells as infection proceeded (Fig. 2c). A similar decline was observed in hr-infected cells but not in wild-type-infected cells when late events were prevented by inhibiting DNA synthesis with cytosine arabinoside (not shown). This implies that protein synthesis did not cease in hr-infected cells, due to the absence of an early to late switch but rather that some other defect in maintaining a high level of protein synthesis was involved. During the first 2 hr after hr infection a number of viral-induced polypeptides of molecular weights similar to those induced in wild-type-infected cells could be detected (Fig. 3A). Thus most early viral polypeptides, further characterized by their appearance in the presence of cytosine arabinoside (Fig. 3B), were synthesized in hr-infected cells. Two early viral-induced polypeptides migrating between 20 and 30K daltons and detected only in the presence of cytosine arabinoside were barely labeled in hr-infected cells. The failure to detect enhanced synthesis of these early polypeptides after hr infection may be a consequence of the rapid inhibi-

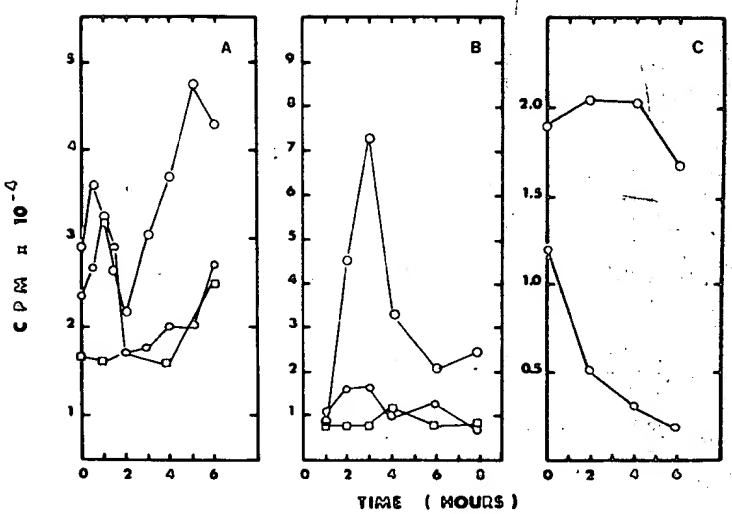


FIG. 2. RNA, DNA, and protein syntheses in KB cells infected with the hr mutant and the wild type. KB cells were infected with 20 PFU per cell (A) or 10 PFU per cell (B and C) either with the hr mutant (○) or the wild type (○). At the indicated times cells were pulse labeled with [^{3}H]uridine for 10 min in (A) with [^{3}H]thymidine for 30 min in (B) or with [^{35}S]methionine for 2 hr in (C). Radioactivity incorporated into RNA (A), DNA (B), and protein (C) was then determined as described under Materials and Methods. (□, mock infection.)

tion of overall protein synthesis that occurs with the hr mutant even in the presence of cytosine arabinoside or to the reduced intensities of all the bands in mutant-infected cells. Another early polypeptide (42K) was apparent in wt-infected cells whereas in hr-infected cells only a background level, that may correspond to a host polypeptide, was observed. The absence of synthesis of this polypeptide has also been noticed in hr infected permissive cells (see below). All polypeptide species appearing late in infection with the wild type were absent in hr-infected cells.

Electron microscopy confirmed that the hr mutant was interrupted in an early stage of infection (Fig. 4). Cytoplasmic foci of infection corresponding to areas devoid of cell organelles and often surrounded by rough endoplasmic reticulum were frequently observed; however, evidence of early assembly products such as membranes or immature particles was not obtained. In some cases dense aggregates which could correspond either to input DNA or to the low level of newly synthesized DNA were found within the viroplasms (not shown).

DNA Deletion in the hr Mutant

The determination of the frequency of reversion of the hr phenotype to the wild-type phenotype should allow a preliminary evaluation as to whether the mutant phenotype is due to a point mutation or a deletion. To investigate this question 1.5×10^7 PFU of the hr mutant were plated on non-permissive cells (KB cell monolayers) at a sufficiently low multiplicity of infection to avoid cell necrosis and enable the visualization of potential revertant plaques. Under these conditions no plaques were obtained suggesting that the mutation was due to a deletion or more unlikely to a point mutation with an extremely low reversion frequency (less than 10^{-7}). Comparison of the DNA from viral particles by velocity sedimentation in neutral sucrose gradients demonstrated that hr DNA was smaller than wt DNA by about 1/10th of the vaccinia genome (results not shown). This was confirmed by agarose gel electrophoretic analysis of viral DNA that had been digested with the restriction enzyme *Hin*dIII (Fig. 5a). The pattern obtained with our wild type corresponded closely to

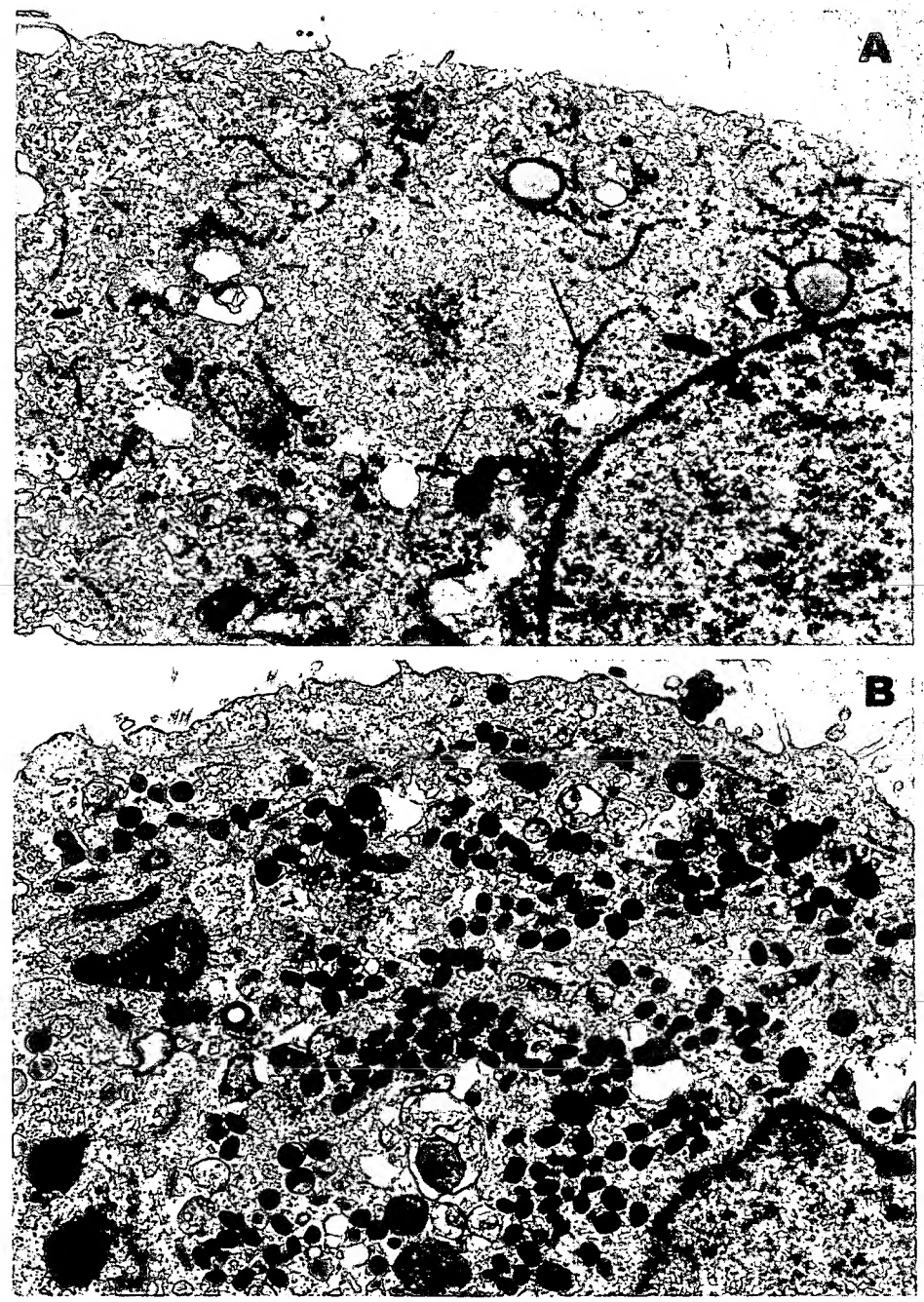
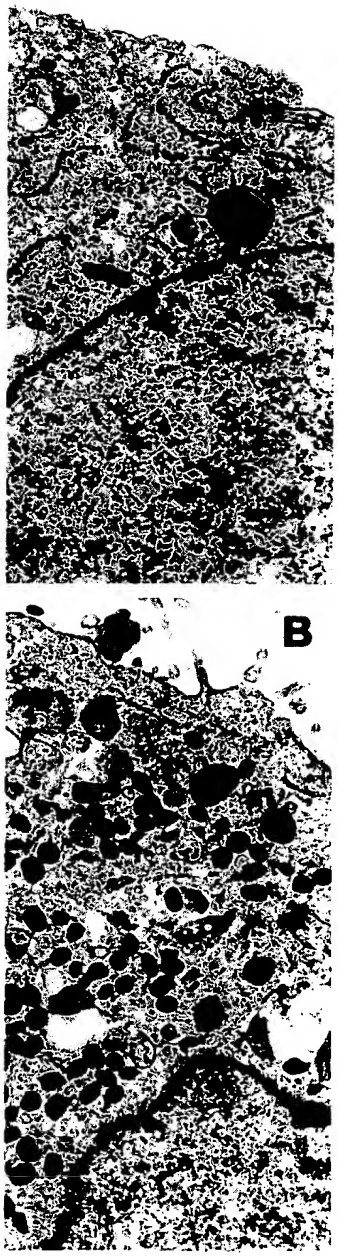


FIG. 4. Electron microscopy of wild-type and hr-infected KB cells. KB cells were infected with 1 PFU per cell for 24 hr either with the hr mutant (A) or the wild type (B) and processed for electron microscopy (× 12,240). A typical viroplasm (V) is apparent in (A) Arrows indicate endoplasmic reticulum that in some cases completely surrounds the viroplasm. In (B) numerous mature viral particles are visible.



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(B) numerous mature viral particles

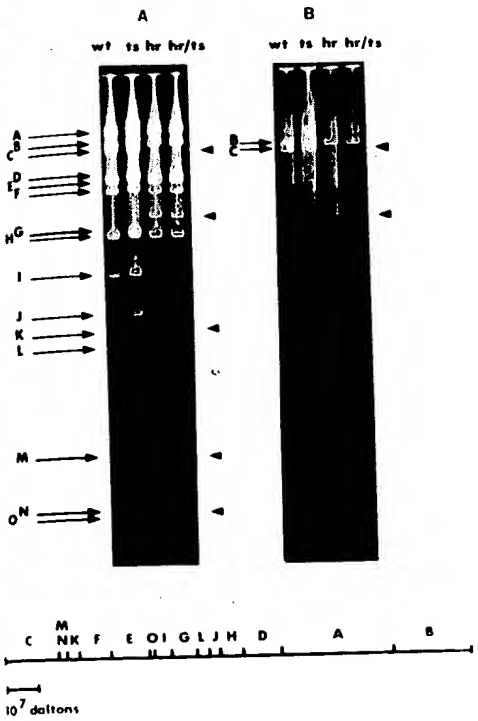


FIG. 5. *Hind*III digestion of vaccinia virus DNA. (A) DNA was digested with *Hind*III and subjected to agarose gel electrophoresis as described under Methods. wt, Wild type; ts, ts_2 mutant; hr, host range mutant; hr/ts, recombinant displaying both host range and ts_2 phenotype. (B) Terminal crosslinked fragments from the same viral strains as in (A) Restriction enzyme digests were denatured and rapidly reannealed before agarose gel electrophoresis as described under Methods. Arrows on the left of (A) and (B) indicate the lettering of fragments in the order of their molecular weights. Arrowheads on the right of (A) and (B) point to modifications (either a new fragment or missing fragments) in the hr and hr/ts mutants. The *Hind*III restriction map below the gel is reproduced from Mackett and Archard (1979).

served mainly the terminal snap back fragments B and C from the wild type while in the hr digest the B fragment and the new fragment migrating between F and G were prominent (Fig. 5b). Thus this fragment is the new crosslinked terminal fragment in hr DNA. The size of the deletion in the hr mutant was estimated to be about 12.6 megadaltons by summing the apparent molecular weights of the various *Hind*III fragments (Table 2).

Lack of Synthesis of an Early Viral-Induced Polypeptide in hr-Infected Permissive Cells

Deletion of DNA in the hr mutant could result in failure to synthesize certain viral polypeptides provided the deleted region is not repetitive information and actually codes for protein. This aspect was investigated by following polypeptide synthesis after infection of permissive BHK cells (Fig. 6a). Most of the viral-induced polypeptides detected in wild-type-infected cells were found in hr-infected cells with similar intensities and at similar periods after infection with one major exception being the 42K species. This polypeptide ap-

TABLE 2

FRAGMENTS PRODUCED BY HindIII DIGESTION OF DNA FROM VACCINIA VIRUS WILD-TYPE AND THE hr MUTANT

Fragment	Apparent molecular weight ($\times 10^{-6}$)	
	Wild type	hr
A	30	30
B	19.5	19.5
C	14	—
D	9.8	9.8
E	9.6	9.6
F	8.7	8.7
F′	—	6.6
G	5.7	5.7
H	5.6	5.6
I	4.2	4.2
J	3.2	3.2
K	2.9	—
L	2.6	2.6
M	1.4	—
N	0.9	—
O	0.8	0.8
Total	118.9	106.3

Note. Molecular weights were determined from the gel shown in Fig. 5 by comparison with a HindIII lambda DNA digest run on the same gel. To facilitate comparison, lettering of the hr fragments corresponding to wt fragments is conserved. The F′ fragment is the new left end terminal crosslinked fragment found in the hr mutant.

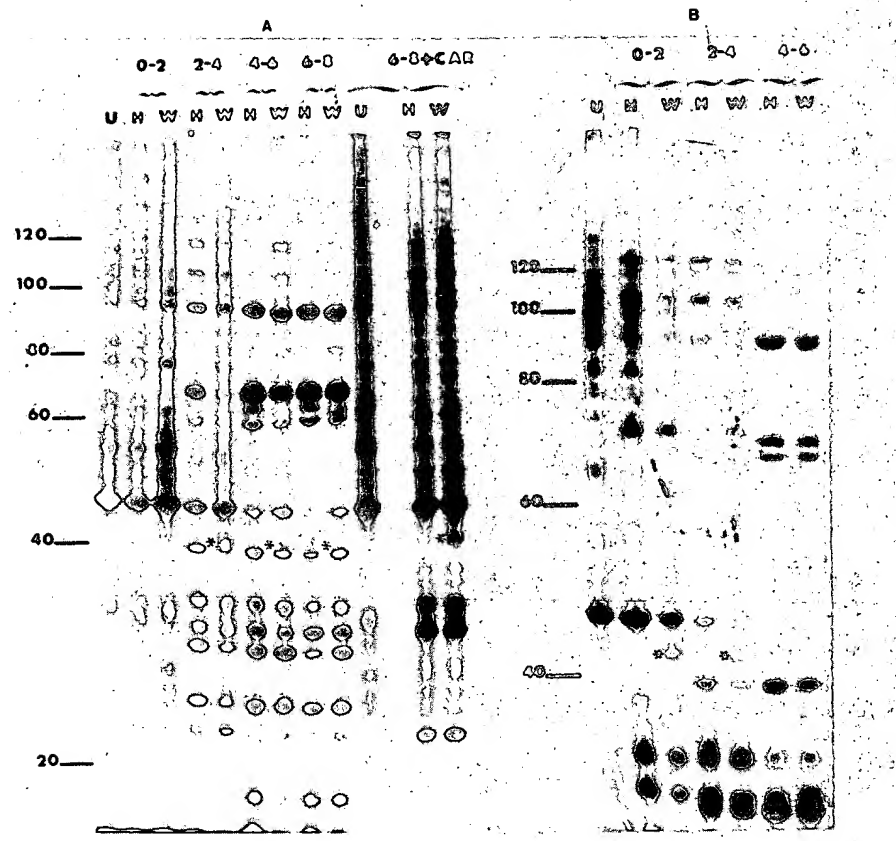
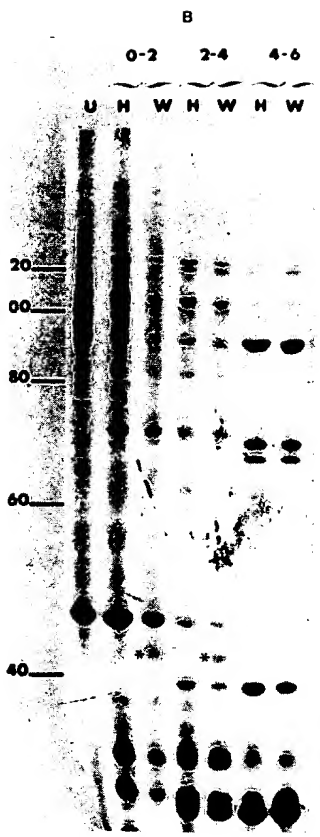


FIG. 6. Polypeptide induction in permissive BHK 21 and semipermissive CV_1 cells after infection with the wild-type or hr mutant. Cells were either mock infected (U) or infected with approximately 10 PFU per cell of the hr mutant (H) or the wild type (W). At the indicated intervals cells were pulse labeled with [^{35}S]methionine. Following labeling cell lysates were submitted to gel electrophoresis as described under Methods. In one case infected cells were incubated in the presence of cytosine arabinoside (CAR 10^{-3} *M*) from the beginning of the infection and labeled at the sixth hour postinfection. (A) BHK 21 cells; (B) CV_1 cells. The scale on the left indicates the apparent molecular weights ($\times 10^{-3}$) of polypeptides. The stars show the position of the 42K polypeptide present in wild-type-infected cells and missing in the hr-infected cells.

peared early after infection with the wild type and its synthesis was shut off at later times. When viral DNA synthesis was inhibited by addition of cytosine arabinoside to the culture medium the 42K polypeptide continued to be synthesized even later in infection with the wild type whereas it was apparently lacking in hr-infected cells (Fig. 6a). The absence of synthesis of the 42K species was also clearly demonstrated in hr-infected CV 1 cells in which no other alteration in polypeptide induction could be detected (Fig. 6b).

Temperature-Sensitive hr Recombinants Retain all hr Properties

If the various characteristics of the hr mutant (plaque morphology, host range, lack of synthesis of the 42K polypeptide) are not all due to the deletion that has been identified, nor to closely linked independent mutations, it should be expected that these phenotypes segregate from one another upon recombination. Therefore, we analyzed the properties of five recombinants obtained between the hr mutant and



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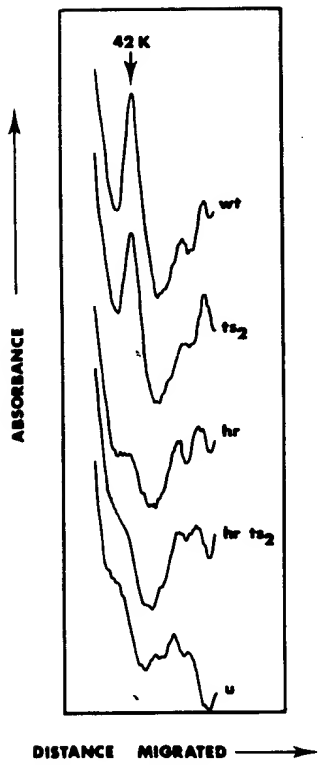

FIG. 7. Induction of the 42K polypeptide after infection with the wild-type or various mutants. BHK 21 cells were infected with 5 PFU per cell in the presence of cytosine arabinoside (10^{-3} *M*). After 6 hr of infection cells were pulse labeled for 2 hr with [^{35}S]methionine and then cell lysates were submitted to gel electrophoresis as described under Methods. The intensities of the bands in the autoradiograms were determined by densitometry with a Vernon spectrophotometer. For clarity only the region flanking the 42K polypeptide is shown. Infection was with the wild type (wt), the ts_2 mutant (ts_2), the hr mutant (hr), a recombinant bearing the ts_2 and the hr mutation (ts_2 hr), and uninfected cells (U).

a previously characterized unlinked temperature sensitive mutant (ts_2). All of the ts_2 hr recombinants selected either for host range or for plaque morphology displayed both host range (failure to multiply in KB or 809 cells) and plaque morphology (small plaques, fusion of CV 1 cells). Moreover all five recombinants failed to synthesize the 42K polypeptide in BHK cells (Fig. 7, result shown for one recombinant) and among the four recombinants tested all exhibited *Hin*dIII restriction patterns identical to the hr mutant (Fig. 5, result shown for one recombinant).

DISCUSSION

The results obtained in this study suggest that the sequences deleted in the hr mutant encode information involved in the determination of both the host range and the cytopathic effect of vaccinia virus. This is supported by the fact that the sorting of genes during recombination between the hr mutant and a ts mutant failed to segregate any of the original hr properties. That such a large deletion encompassing 10% of the genome results in a variety of phenotypes is not surprising. Interestingly enough complete host range restriction of the mutant seems to be limited as far as tested to human cells. The only exception being some of the human embryo cells which were found to be semipermissive. Preliminary results suggest that this corresponds to the presence of a small number of permissive cells within the population rather than to the semipermissiveness of all cells. Monkey cells, the most closely related phylogenetically to human cells, were semipermissive. Therefore it is possible that the hr mutation affects genes that are required specifically for efficient multiplication of vaccinia virus in primate cells although further work is necessary to establish this.

Abortiveness of the hr mutant in human KB cells appears to be due to a defect either in the stability of early viral mRNA or at the level of translation. Thus, although early viral mRNA and protein synthesis occur to a certain extent the very rapid decline in overall protein synthesis implies the arrest of hr gene expression. In this respect the hr mutant behaves in human cells much as our wild-type vaccinia virus in chinese hamster ovary cells (Drillien *et al.*, 1978b). On the basis of electron microscopy the hr mutant is similar to the group B temperature sensitive mutants described by Dales *et al.* (1978).

The lack of synthesis of the early 42K polypeptide in BHK and CV 1 cells indicates that this polypeptide is nonessential

in cells permissive for the hr mutant. It is likely that the gene encoding the 42K species maps within the hr-deleted sequences. Wittek *et al.* (1980) have recently shown that the vaccinia virus-inverted terminal repeats of approximately 6.8×10^6 daltons in length encode among several other proteins an immediate early 42K polypeptide. If this polypeptide is identical to the one missing in hr-infected cells then the hr deletion presumably overlaps the left-hand terminal repeat. In this case either the right-hand end also contains an as yet undetected symmetrical deletion in the terminal repeat as has been found for some spontaneously arising vaccinia mutants (McFadden and Dales, 1979) or the right-hand sequence remains silent. The fact that a deletion of 12.6×10^6 daltons of DNA results in the absence of synthesis of only a single polypeptide may be accounted for by the detection of only a fraction of the viral-induced polypeptides. It is expected that the use of higher resolution methods should allow the identification of other minor polypeptide species missing in infections with the hr mutant.

Extensive deletions in the terminal sequences of mutants of the closely related rabbit pox virus have been previously described (Moyer and Rothe, 1980; Lake and Cooper, 1980). The white pock host range mutants contain spontaneous deletions mapping in the left-hand end where they may overlap the terminal repeat and encompass from 3 to 20×10^6 daltons (Moyer and Rothe, 1980). In the white pock u mutants deletions were detected in the right-hand end (Lake and Cooper, 1980). In a white pock mutant of the red cowpox virus a deletion in the right-hand end of 16 to 18×10^6 daltons including the entire terminal repeat has been described (Archard and Mackett, 1979). White pock mutants of the monkeypox virus have also recently been shown to be either extensive deletions at the right-hand end or complex rearrangements involving DNA insertions at both ends (Dumbell and Archard, 1980). Our results demonstrate that vaccinia virus like the other poxviruses mentioned above contains a considerable amount of dispensable information that can include as much as 10% of the genome and further support the notion that terminal sequences in the pox viruses determine host range and various cytopathic effects.

ACKNOWLEDGMENTS

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REFERENCES

ARCHARD, L. C., and MACKETT, M. (1979). Restriction endonuclease analysis of red cowpox virus and its white pock variant. *J. Gen. Virol.* 45, 51–63.

BONNER, W. M., and LASKEY, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46, 83–88.

DALES, S., MILOVANOVITCH, V., POGO, B. G. T., WEINTRAUB, S. B., HUIMA, T., WILTON, S., and MCFADDEN, G. (1978). Biogenesis of vaccinia: Isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. *Virology* 84, 403–428.

DRILLIEN, R., TRIPIER, F., KOEHREN, F., and KIRN, A. (1977). A temperature sensitive mutant of vaccinia virus defective in an early stage of morphogenesis. *Virology* 79, 369–380.

DRILLIEN, R., KELLER, F., KOEHREN, F., and KIRN, A. (1978a). Ts mutants of vaccinia virus: Biochemical and biological defectiveness. *In* "Viruses and Environment" (E. Kurstak and K. Maramorosch, eds.), pp. 381–396. Academic Press, New York.

DRILLIEN, R., SPEHNER, D., and KIRN, A. (1978b). Host range restriction of vaccinia virus in chinese hamster ovary cells: Relationship to shut off of protein synthesis *J. Virol.* 28, 843–850.

DUMBELL, K., and ARCHARD, L. C. (1980). Comparison of white pock (h) mutants of monkeypox virus with parental monkeypox and variola-like viruses isolated from animals. *Nature (London)* 286, 29–32.

FENNER, F., and SAMBROOK, J. F. (1966). Conditional lethal mutants of rabbitpox virus. II Mutants (p) that fail to multiply in PK-2a cells. *Virology* 28, 600–609.

FRIED, M. (1965). Isolation of temperature sensitive mutants of polyoma virus. *Virology* 25, 669–671.

GANGEMI, J. D., and SHARP, D. G. (1978). Particle heterogeneity in vaccinia virus population during passage. *Virology* 85, 262–270.

GEMMELL, A., and FENNER, F. (1960).

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REFERENCES

:D, L. C., and MACKETT, M. (1979). Restric-
ndonuclease analysis of red cowpox virus and
ite pock variant. *J. Gen. Virol.* 45, 51–63.
:, W. M., and LASKEY, R. A. (1974). A film de-
ı method for tritium-labelled proteins and nu-
cids in polyacrylamide gels. *Eur. J. Biochem.*
–88.
S., MILOVANOVITCH, V., POGO, B. G. T.,
RAUB, S. B., HUIMA, T., WILTON, S., and
DDEN, G. (1978). Biogenesis of vaccinia: Isola-
f conditional lethal mutants and electron mi-
pic characterization of their phenotypically
ised defects. *Virology* 84, 403–428.
N, R., TRIPIER, F., KOEHREN, F., and KIRN,
77). A temperature sensitive mutant of vac-
irus defective in an early stage of morphogen-
irology 79, 369–380.
N, R., KELLER, F., KOEHREN, F., and KIRN,
78a). Ts mutants of vaccinia virus: Biochemi-
l biological defectiveness. *In* "Viruses and
nment" (E. Kurstak and K. Maramorosch,
pp. 381–396. Academic Press, New York.
N, R., SPEHNER, D., and KIRN, A. (1978b).
ange restriction of vaccinia virus in chinese
r ovary cells: Relationship to shut off of pro-
nthesis *J. Virol.* 28, 843–850.
L, K., and ARCHARD, L. C. (1980). Compari-
white pock (h) mutants of monkeypox virus
arental monkeypox and variola-like viruses
l from animals. *Nature (London)* 286, 29–32.
F., and SAMBROOK, J. F. (1966). Conditional
mutants of rabbitpox virus. II Mutants (p)
il to multiply in PK-2a cells. *Virology* 28,
9.
I. (1965). Isolation of temperature sensitive
s of polyoma virus. *Virology* 25, 669–671.
, J. D., and SHARP, D. G. (1978). Particle
geneity in vaccinia virus population during
. *Virology* 85, 262–270.
., A., and FENNER, F. (1960).

Genetic studies with mammalian poxviruses. III
White (u) mutants of rabbit pox virus. *Virology* 11,
219–235.
GESHLIN, P., and BERNS, K. I. (1974).
Characterization and localization of the naturally oc-
curring cross-links in vaccinia virus DNA. *J. Mol.
Biol.* 88, 785–796.
LAEMMLI, U. K. (1970). Cleavage of structural pro-
teins during assembly of the head of bacteriophage
T_4. *Nature (London)* 227, 680–685.
LAKE, J. R., and COOPER, P. D. (1980). Deletions of
the terminal sequences in the genome of the white
pock (u) and host restricted (p) mutants of rabbitpox
virus. *J. Gen. Virol.* 48, 135–147.
MCCLAIN, M. E. (1965). The host range and plaque
morphology of rabbitpox virus (RPu^+) and its u mu-
tants on chick fibroblast PK-2a and L 929 cells.
Aust. J. Exp. Biol. Med. Sci. 43, 31–44.
MCFADDEN, G., and DALES, S. (1979). Biogenesis of
poxviruses: Mirror-image deletions in vaccinia virus
DNA. *Cell* 18, 101–108.
MACKETT, M., and ARCHARD, L. C. (1979). Conserva-
tion and variation in orthopoxvirus genome struc-
ture. *J. Gen. Virol.* 45, 683–701.
MOSS, B. (1974). Reproduction of poxviruses in "Com-
prehensive Virology" (H. Fraenkel-Conrat and R.
R. Wagner, eds.), Vol. 3, pp. 405–474. Plenum,
New York.
MOYER, R. W., and ROTHE, C. T. (1980). The white
pock mutants of rabbit poxvirus. I. Spontaneous
host range mutants contain deletions. *Virology* 102,
119–132.
PARKHURST, J. R., and HEIDELBERGER, C. (1976).
Rapid lysis of vaccinia virus on neutral sucrose gra-
dients with release of intact DNA. *Anal. Biochem.*
71, 53–59.
SAMBROOK, J. F., PADGETT, B. L., and TOMKINS, J.
K. N. (1966). Conditional lethal mutants of rabbit-
pox virus. I. Isolation of host-cell-dependent and
temperature dependent mutants. *Virology*, 28,
592–599.
STUDIER, F. W. (1973). Analysis of bacteriophage T_7
early RNAs and proteins on slab gels. *J. Mol. Biol.*
79, 237–248.
TAGAYA, I., KITAMURA, T., and SANO, Y. (1961). A
new mutant of dermovaccinia virus. Nature (Lon-
don) *192*, 381–382.
WITTEK, R., BARBOSA, E., COOPER, J. A., GARON, C.
F., CHAN, H., and MOSS, B. (1980). Inverted termi-
nal repetition in vaccinia virus DNA encodes early
mRNAs. *Nature (London)* 285, 21–25.

Journal of General Virology (1991), 72, 699–703. Printed in Great Britain

Homotypic and heterotypic protection against influenza virus infection in mice by recombinant vaccinia virus expressing the haemagglutinin or nucleoprotein gene of influenza virus

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Recombinant vaccinia virus expressing the influenza virus haemagglutinin (HA) or nucleoprotein (NP) genes from A/SW/Hong Kong/1/74 (H1N1) under the control of a hybrid promoter containing the P7.5 early promoter element and promoter of the gene encoding the major protein of cowpox virus A type inclusion body was constructed to investigate protective immunity against homologous and heterologous viruses in mice. These recombinant vaccinia viruses produced authentic influenza virus HA and NP in infected cells. The recombinant vaccinia virus–influenza virus HA conferred efficient subtype-specific protection although mice challenged with heterologous influenza viruses underwent initial infection. By contrast, immunization with the recombinant vaccinia–influenza virus NP limited virus multiplication in the lungs against challenge infection with all H1N1 and H3N2 influenza viruses examined, although less efficiently. These results will prompt the re-examination of the possibility of using the recombinant vaccinia virus–influenza virus NP as a cross-protective vaccine

Antibody-mediated immunity to influenza virus is strain- or subtype-specific, whereas cell-mediated immunity is cross-reactive among type A influenza viruses (Askonas *et al.*, 1982; Ada *et al.*, 1983; Ada & Jones, 1986). The major protective antibody is directed against the envelope glycoprotein haemagglutinin (HA). Influenza virus HA-specific antibody neutralizes infectivity of the virus *in vitro* and protects mice from challenge infection with homologous virus (Askonas *et al.*, 1982). On the other hand, cytotoxic T lymphocytes (CTL) recognize the nucleoprotein (NP) as a cross-reactive antigen and aid virus clearance from the host (Ennis *et al.*, 1978; Yewdell *et al.*, 1985; Taylor & Askonas, 1986; McMichael *et al.*, 1986; Gotch *et al.*, 1987). Previous studies demonstrated that recombinant vaccinia virus expressing influenza virus HA elicits virus-neutralizing antibody and subtype-specific CTL (Panicali *et al.*, 1983; Smith *et al.*, 1983; Bennink *et al.*, 1984; Coupar *et al.*, 1986), and that recombinants expressing NP stimulate cross-reactive CTL (Yewdell *et al.*, 1985). The protective efficacy of HA and NP against homologous virus has been evaluated; vaccinia–HA recombinant virus affords total protection against lethal challenge infection, but vaccinia–NP virus provides poor protective immunity (Andrew *et al.*, 1987; Andrew & Coupar, 1988; Stitz *et al.*, 1990). Since the antigenicity of influenza virus changes with time, protection against heterologous virus is of considerable importance for the development of an influenza vaccine.

In this study, we constructed new recombinant vaccinia virus expressing either the influenza virus HA or NP gene under the control of a hybrid cowpox–vaccinia virus promoter with enhanced promoter activity and have investigated the protective efficacy of these two recombinant vaccinia viruses against challenge infection with homologous and heterologous influenza viruses.

To construct the recombinant vaccinia viruses, full-length cDNA was synthesized from purified viral RNA of A/SW/Hong Kong/1/74 (SW/HK) (H1N1) by using the universal primer 5′AGCAAAAGCAGG complementary to the 3′ terminus of viral RNA. The cDNA of either the HA or the NP gene was ligated with an *Eco*RI linker and inserted into the *Eco*RI site of plasmid pSFB5, which contains the flanking HA gene sequence of vaccinia virus for homologous recombination with the

vaccinia virus genome at the HA locus and four unique sites for the insertion of a foreign gene downstream from a hybrid cowpox–vaccinia virus promoter. The resultant plasmids, pSHA1 and pSNP1, were used to generate recombinant viruses vSHA1 (HA-expressing recombinant virus) and vSNP1 (NP-expressing virus), respectively, as described previously (Itamura *et al.*, 1990*a*). The protein expressed from the recombinant vaccinia viruses was analysed using a radioimmunoprecipitation assay with antiserum specific for SW/HK virus (Fig. 1). A 74K polypeptide was detected in vSHA1-infected cells, and it appeared similar in size to the glycosylated, uncleaved influenza virus HA. In vSNP1-infected cells, a 56K polypeptide was observed, corresponding to the expected size of influenza virus NP. Immunofluorescence studies indicated that the HA expressed from vSHA1 was transported to the cell surface and, in contrast, the NP expressed from vSNP1 was predominantly localized in the nucleus (data not shown). We observed haemadsorption of chicken erythrocytes and trypsin-activated, acid-inducible cell fusion by vSHA1-infected cells, but not by vSNP1-infected cells (data not shown). Thus the recombinant vaccinia viruses were found to express functionally authentic influenza virus HA from vSHA1 and NP from vSNP1.

To examine the protective ability of the recombinant vaccinia viruses against homologous virus, groups of ddY mice were immunized intravenously (i.v.) with 10^7 p.f.u. of either vSHA1, vSNP1 or insert-free vaccinia virus WR(HA$^-$), and 21 days later were challenged with an aerosol mist of SW/HK virus by using a jet nebulizer (Nihon Clea). In this experiment, no group of mice showed any symptoms of morbidity or pathological changes in the lung. Table 1 shows virus multiplication in the lungs of immunized mice after challenge with SW/HK virus. All mice immunized with vSHA1 were completely protected from infection. By contrast, immunization with vSNP1 did not protect mice from infection but reduced the lung virus titre on day 7 when compared with the immunization effect of WR(HA$^-$), although its efficiency was lower than that with vSHA1. We also examined serum neutralizing antibody titres against SW/HK virus in vaccinated mice prior to challenge infection (Table 1). The recombinant virus vSHA1 induced high levels of serum neutralizing antibody in mice but vSNP1 and WR(HA$^-$) did not produce detectable levels.

To analyse the efficacy of heterotypic protection by recombinant vaccinia viruses, we used two heterologous viruses, A/Yamagata/120/86 (Yam) (H1N1) and A/Sichuan/2/87 (Sic) (H3N2) as challenge viruses. Although the heterologous virus Yam belongs to the same serotype as the homologous SW/HK virus, it is antigenically distinct. The ddY mice (11 per group) immunized with

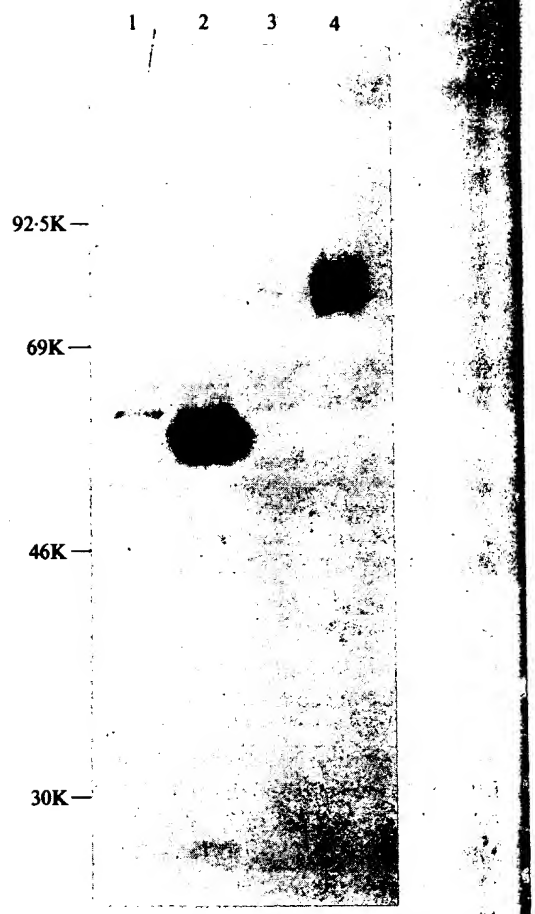

Fig. 1. Radioimmunoprecipitation analysis of protein expressed from vSHA1 and vSNP1. CV-1 cells were infected at 10 p.f.u./cell with vSNP1 (lanes 1 and 2) or vSHA1 (lanes 3 and 4); 4 h post-infection (p.i.) the infected cells were labelled with 50 μCi of [^{35}S]methionine (100 μCi/ml). At 15 h p.i., cells were disrupted and the lysate was used in an immunoprecipitation reaction with normal rabbit serum (lanes 1 and 3) or rabbit anti-SW/HK virus serum (lanes 2 and 4), as described previously (Itamura *et al.*, 1990*a*). Immunoprecipitates were electrophoresed on a 12% polyacrylamide gel containing SDS and fluorographed. M_r markers are indicated on the left.

vSHA1, vSNP1 or WR(HA$^-$) were challenged with an aerosol mist of 3 LD_{50} Yam virus 21 days post-immunization. All mice immunized with vSHA1 survived the lethal challenge but showed some signs of morbidity such as lethargy, hunching, weight loss and rapid respiration. Immunization with vSNP1 partially protected mice from the lethal challenge (73%) and caused morbidity in all mice according to the above criteria. Almost all mice (82%) immunized with WR(HA$^-$) had died by day 12 with this challenge dose. The weight loss in mice immunized with vSHA1 was recovered earlier than that in mice protected by vSNP1 (data not shown). The challenge virus was detected in the lungs of all mice immunized with vSHA1, vSNP1 or WR(HA$^-$) on days 3 and 5 (Table 1). However,

Table 1. *Protection from challenge infection of influenza viruses in mice* immunized with recombinant vaccinia viruses*

Challenged with	Vaccinated with	Neutralizing† antibody titre	Pulmonary virus titre‡ (log_{10} p.f.u./ml) day 3	day 5	day 7
SW/HK (H1N1)	vSHA1	8192	<2·0	<2·0	<2·0
	vSNP1	<32	5·4	4·7	2·7
	WR(HA⁻)	<32	5·3	5·1	3·7
Yam (H1N1)	vSHA1	32	6·2	4·8	<2·0
	vSNP1	<32	6·3	5·9	2·7
	WR(HA⁻)	<32	7·0	6·2	4·8
Sic (H3N2)	vSHA1	<32	6·2	6·0	4·4
	vSNP1	<32	6·0	5·9	<2·0
	WR(HA⁻)	<32	6·1	5·9	4·8

* ddY outbred mice (obtained from Japan SLC) were challenged with the indicated virus 21 days after immunization with the recombinant or the control WR(HA⁻).

† Neutralizing antibody titres against challenge viruses were determined in pooled sera from four to six immunized mice prior to challenge using the 50% reduction of plaque formation assay. The neutralizing antibody titre was expressed as the reciprocal of the serum dilution causing 50% reduction of plaque formation.

‡ Homogenates (2 ml/lung) of three pooled lungs per group were titrated on MDCK monolayer cells essentially as described previously (Tobita *et al.*, 1975).

multiplication of the challenge virus in the lung was reduced on days 5 and 7 in mice immunized with vSHA1 and vSNP1. The rate of reduction of lung virus titre in vSHA1-immunized mice was greater than that in vSNP1-immunized mice. vSHA1-immunized mice had low titres of serum neutralizing antibody against Yam, but those immunized with vSNP1 and WR(HA⁻) had no detectable levels of antibody. Similar protection was observed when mice were challenged with a low lethal dose of A/PR/8/34 (H1N1) virus which is also antigenically distinct from the homologous SW/HK virus (data not shown).

Next, immunized mice were challenged with Sic, which has a different serotype, as described above. In contrast to the protection from challenge with Yam virus, mice immunized with vSHA1 failed to reduce the lung virus titre when compared with WR(HA⁻)-immunized mice (Table 1). However, immunization with vSNP1 was still effective in terms of reduction of the virus in the lung on day 7. Serum neutralizing antibody against Sic virus was not detected in any mice immunized with vSHA1, vSNP1 or WR(HA⁻).

To elucidate the mediator of protection from virus multiplication in the lung of mice immunized with vSHA1 or vSNP1, we analysed the ability of the recombinant vaccinia viruses vSHA1 and vSNP1 to prime an influenza-specific CTL response. BALB/c (H-2^d) mice were immunized i.v. with 10^7 p.f.u. of the recombinant vaccinia virus. Two weeks later, their spleens were removed and restimulated *in vitro* with SW/HK-infected splenocytes from BALB/c mice. Effector cells were harvested after 5 days and assayed for specific cytotoxicity using a ^{51}Cr release assay as described previously (Itamura *et al.*, 1990*b*). Both the vSHA1 and vSNP1 primed subtype-specific CTL and strong cross-reactive CTL, respectively (Table 2).

Immunization with vSHA1 expressing influenza virus HA provided efficient protection from challenge infection in a subtype-specific manner. Challenge infection with the homologous virus was completely abolished by immunization with vSHA1, whereas immunization with vSHA1 did not protect mice from infection with heterologous H1N1 viruses although it reduced the lung virus titre. Amino acid sequence identities of HA (HA1 coding region) between SW/HK and Yam, and SW/HK and PR8 are 74·8% and 80·4% (Sugita *et al.*, 1991; A. Endo, unpublished results). Similar subtype-specific protection by the recombinant vaccinia virus or fowlpox virus expressing avian influenza virus HA (H5) has been reported previously and the lowest sequence identity between vaccinated and challenge virus HA is still 85% (Chambers *et al.*, 1988; Taylor *et al.*, 1988).

Our results demonstrate the widest range of heterotypic protection. Since immunization with vSHA1 completely protected the lung from challenge infection with the homologous virus and produced high levels of virus-neutralizing antibody, the most plausible mediator of protection against the homologous virus is virus-neutralizing antibody. Undetectable levels of neutralizing antibody have been demonstrated to be valid for protection against challenge infection with influenza virus (Chambers *et al.*, 1988; Andrew & Coupar, 1988;

Table 2. *Ability of recombinant vaccinia viruses to prime influenza virus-specific CTL response*

Primed with	E/T*	Specific cytotoxicity (%) to target cells infected with the following virus†				
		SW/HK (H1N1)	Yam (H1N1)	Sic (H3N2)	B/Yam	Mock
vSHA1	80	34·9	22·8	10·4	9·4	10·5
	40	27·3	17·3	7·1	4·3	7·9
	20	15·5	14·1	4·1	1·1	7·1
vSNP1	80	46·4	42·3	21·0	4·0	8·7
	40	34·1	33·4	16·0	1·4	8·6
	20	28·4	19·2	5·1	2·1	3·3

* E/T, Effector-to-target ratio.

† Cytotoxic activity of secondary *in vitro* stimulated splenocytes from BALB/c mice primed with vSHA1 or vSNP1 were measured against mastocytoma P815 (H-2^d) cells infected with influenza viruses, SW/HK, Yam, Sic and B/Yamagata/16/88 (B/Yam) or uninfected (Mock) cells. Cytotoxicity was expressed as the percentage of specific ^{51}Cr release from target cells.

Hunt *et al.*, 1988). Therefore, virus-neutralizing antibody also may play an important role, even against heterologous H1N1 viruses, in the reduction of lung virus titre. In addition, CTL also may contribute to clearance of heterologous H1N1 viruses from the host.

Immunization with vSNP1 expressing influenza virus NP reduced virus multiplication in the lungs of mice challenged with all type A influenza viruses examined, but it was less efficient although more cross-protective than immunization with vSHA1. Immunization with vSNP1 did not reduce the initial rate of replication in challenged mice and did not induce detectable neutralizing antibody, but it primed a cross-reactive CTL response against type A influenza viruses. These findings suggest that the protection conferred by vSNP1 is mediated by cross-reactive CTL. The above speculation is also supported by the following previous findings: (i) NP-specific antibody has no virus-neutralizing activity (Virelizier *et al.*, 1979), (ii) CTLs aid in limiting viral replication and dissemination (Ennis *et al.*, 1978), (iii) NP is a major target antigen for cross-reactive anti-influenza A virus CTLs (Yewdell *et al.*, 1985) and (iv) adoptive transfer of NP-specific CTL clones confers cross-protection from lethal challenge (Taylor & Askonas, 1986). Previous studies showed that mice vaccinated with the recombinant vaccinia virus expressing NP are poorly protected from mortality and morbidity (Andrew *et al.*, 1987; Andrew & Coupar, 1988; Stitz *et al.*, 1990). Reduction of lung virus titre by vSNP1 seems to be more efficient than that by the recombinant vaccinia virus reported previously (Andrew *et al.*, 1987).

The difference in protective efficacy may be related to the insertion site in the vaccinia virus genome, the vaccinia virus promoter or the molecular species of the NP gene used for the construction of recombinant vaccinia virus. In our recombinant viruses, we used the HA gene locus as an insertion site, the NP gene of SW/HK virus and the new ATI–P7.5 hybrid promoter (containing the P7.5 early promoter element and the promoter of the gene encoding the major protein of cowpox virus A type inclusion body), which directed enhanced expression at both early and late phases of infection (S. Funahashi & H. Shida, unpublished results). In a previous study, the recombinant vaccinia virus was constructed to express the influenza NP gene of A/PR/8/34 (H1N1) virus under the control of the P7.5 promoter from the thymidine kinase (TK) gene locus (Andrew *et al.*, 1987). Indeed, we observed the effect of insertion sites (vaccinia virus TK and HA gene loci) on the ability of recombinant vaccinia viruses to prime an influenza virus H3-specific CTL response (Itamura *et al.*, 1990*b*). Recent studies also revealed that induction of CTL is profoundly affected by the molecular species expressed from recombinant vaccinia virus, by its temporal expression and its degradation (Coupar *et al.*, 1986; Townsend *et al.*, 1988).

Influenza viruses change antigenically with time in an unpredictable way; therefore, a vaccine able to elicit cross-reactive immunity would be of advantage. Although it has been suggested that NP is a candidate for vaccines promoting cross-reactive immunity, others have shown that immunization with the NP-recombinant virus alone is insufficient to protect against challenge (Andrew & Coupar, 1988; Stitz *et al.*, 1990). Our data may however encourage further improvement of recombinant vaccinia virus expressing NP.

In mice infected with influenza virus, morbidity and mortality occur as immunopathological consequences rather than a direct effect of virus multiplication (Singer *et al.*, 1972; Hurd & Heath, 1975; Sullivan *et al.*, 1976). NP-recombinant virus limited virus propagation in the lung although it could not prevent infection and disease. Therefore, partially cross-protective NP-recombinant virus may be beneficial in the prevention of fatal pneumonia in man. Alteration of the vaccinia virus promoter and/or co-expression of lymphokine genes such as interleukin 2 or interferon-γ may increase the level of cross-reactive immunity obtainable by NP-recombinant vaccinia virus (Coupar *et al.*, 1986; Yilma *et al.*, 1987; Flexner *et al.*, 1988; Davison & Moss, 1989).

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References

ADA, G. L. & JONES, P. D. (1986). The immune response to influenza infection. *Current Topics in Microbiology and Immunology* **128**, 1–54.

ADA, G. L., MAK, N. K. & SWEET, C. (1983). The regulation of influenza virus infection. *Progress in Immunology* **5**, 1295–1304.

ANDREW, M. E. & COUPAR, B. E. H. (1988). Efficacy of influenza haemagglutinin and nucleoprotein as protective antigens against influenza virus infection in mice. *Scandinavian Journal of Immunology* **28**, 81–85.

ANDREW, M. E., COUPAR, B. E. H., BOYLE, D. B. & ADA, G. L. (1987). The roles of influenza virus haemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. *Scandinavian Journal of Immunology* **25**, 21–28.

ASKONAS, B. A., MCMICHAEL, A. J. & WEBSTER, R. G. (1982). The immune response to influenza viruses and the problem of protection against infection. In *Basic and Applied Influenza Research*, pp. 159–188. Edited by A. S. Beare. Boca Raton: CRC Press.

BENNINK, J. R., YEWDELL, J. W., SMITH, G. L., MOLLER, C. & MOSS, B. (1984). Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. *Nature, London* **311**, 578–579.

CHAMBERS, T. M., KAWAOKA, Y. & WEBSTER, R. G. (1988). Protection of chicken from lethal influenza infection by vaccinia-expressed hemagglutinin. *Virology* **167**, 414–421.

COUPAR, B. E. H., ANDREW, M. E., BOTH, G. W. & BOYLE, D. B. (1986). Temporal regulation of influenza haemagglutinin expression in vaccinia virus recombinants and effects on the immune response. *European Journal of Immunology* **16**, 1479–1487.

DAVISON, A. J. & MOSS, B. (1989). Structure of vaccinia virus late promoters. *Journal of Molecular Biology* **210**, 771–784.

ENNIS, F. A., WELLS, M. A., BUTCHKO, G. M. & ALBRECHT, P. (1978). Evidence that cytotoxic T cells are part of the host's response to influenza pneumonia. *Journal of Experimental Medicine* **148**, 1241–1250.

FLEXNER, C., HUGIN, A. & MOSS, B. (1988). Attenuation of live recombinant vaccinia virus vectors by expression of human interleukin-2. In *Vaccines '88*, pp. 179–184. Edited by H. Ginsberg, F. Brown, R. A. Lerner & R. M. Chanock. New York: Cold Spring Harbor Laboratory.

GOTCH, F., MCMICHAEL, A., SMITH, G. L. & MOSS, B. (1987). Identification of viral molecules recognised by influenza-specific human cytotoxic T lymphocytes. *Journal of Experimental Medicine* **165**, 408–416.

HUNT, L. A., BROWN, D. W., ROBINSON, H. L., NAEVE, C. W. & WEBSTER, R. G. (1988). Retrovirus-expressed hemagglutinin protects against lethal influenza virus infections. *Journal of Virology* **62**, 3014–3019.

HURD, J. & HEATH, R. B. (1975). Effect of cyclophosphamide on infections in mice caused by virulent and avirulent strains of influenza virus. *Infection and Immunity* **11**, 886–889.

ITAMURA, S., MORIKAWA, Y., SHIDA, H., NEROME, K. & OYA, A. (1990*a*). Biological and immunological characterization of influenza virus haemagglutinin expressed from the haemagglutinin locus of vaccinia virus. *Journal of General Virology* **71**, 1293–1301.

ITAMURA, S., IINUMA, H., SHIDA, H., MORIKAWA, Y., NEROME, K. & OYA, A. (1990*b*). Characterization of antibody and cytotoxic T lymphocyte responses to human influenza virus H3 haemagglutinin expressed from the haemagglutinin locus of vaccinia virus. *Journal of General Virology* **71**, 2859–2865.

MCMICHAEL, A. J., MICHIE, C. A., GOTCH, F. M., SMITH, G. L. & MOSS, B. (1986). Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. *Journal of General Virology* **67**, 719–726.

PANICALI, D., DAVIS, S. W., WEINBERG, R. L. & PAOLETTI, E. (1983). Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proceedings of the National Academy of Sciences, U.S.A.* **80**, 5364–5368.

SINGER, S. H., NOGUCHI, P. & KIRSCHSTEIN, R. L. (1972). Respiratory diseases in cyclophosphamide-treated mice. II. Decreased virulence of PR8 influenza virus. *Infection and Immunity* **5**, 957–960.

SMITH, G. L., MURPHY, B. R. & MOSS, B. (1983). Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proceedings of the National Academy of Sciences, U.S.A.* **80**, 7155–7159.

STITZ, L., SCHMITZ, C., BINDER, D., ZINKERNAGEL, R., PAOLETTI, E. & BECHT, H. (1990). Characterization and immunological properties of influenza A virus nucleoprotein (NP): cell-associated NP isolated from infected cells or viral NP expressed by vaccinia recombinant virus do not confer protection. *Journal of General Virology* **71**, 1169–1179.

SUGITA, S., YOSHIOKA, Y., ITAMURA, S., KANEGAE, Y., OGUCHI, K., GOJOBORI, T., NEROME, K. & OYA, A. (1991). Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. *Journal of Molecular Evolution* (in press).

SULLIVAN, J. L., MAYNER, R. E., BARRY, D. W. & ENNIS, F. A. (1976). Influenza virus infection in nude mice. *Journal of Infectious Diseases* **133**, 91–94.

TAYLOR, P. M. & ASKONAS, B. A. (1986). Influenza nucleoprotein-specific cytotoxic T-cell clones are protective *in vivo*. *Immunology* **58**, 417–420.

TAYLOR, J., WEINBERG, R., KAWAOKA, Y., WEBSTER, R. G. & PAOLETTI, E. (1988). Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine* **6**, 504–508.

TOBITA, K., SUGIURA, A., ENOMOTO, C. & FURUYAMA, M. (1975). Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Medical Microbiology and Immunology* **162**, 9–14.

TOWNSEND, A., BASTIN, J., GOULD, K., BROWNLEE, G., ANDREW, M., COUPAR, B., BOYLE, D., CHAN, S. & SMITH, G, (1988). Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *Journal of Experimental Medicine* **168**, 1211–1224.

VIRELIZIER, J. L., ALLISON, A. C. & SCHILD, G. C. (1979). Immune responses to influenza virus in the mouse and their role in control of infection. *British Medical Bulletin* **35**, 65–68.

YEWDELL, J. W., BENNINK, J. R., SMITH, G. L. & MOSS, B. (1985). Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences, U.S.A.* **82**, 1785–1789.

YILMA, T., ANDERSON, K., BRECHLING, K. & MOSS, B. (1987). Expression of an adjuvant gene (interferon-γ) in infectious vaccinia virus recombinants. *In Vaccines '87*, pp. 393–396. Edited by R.M Chanock, R. A. Lerner, F. Brown & H. Ginsberg. New York: Cold Spring Harbor Laboratory.

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Cloning and Expression of Foreign Genes in Vaccinia Virus, Using a Host Range Selection System

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A simple selection system has been developed for the cloning and expression of open reading frames in vaccinia virus. The selection system is based on a conditional lethal (host range) mutant of vaccinia virus. A deletion mutant of the vaccinia virus WR strain was generated by insertion of the neomycin resistance gene from transposon Tn5 and selection with the antibiotic G418. This deletion recombinant, vP293, lacked approximately 21.7 kilobases of DNA beginning 3.8 kilobases from the left end of the genome. vP293 was capable of plaquing on primary chicken embryo fibroblasts and two monkey cell lines (BSC-40 and Vero) but was defective in replication in the human cell line MRC-5. Insertion of the host range gene K1L into vP293 restored the ability to grow on MRC-5 cells. A series of plasmids were constructed which in addition to the K1L gene contained a vaccinia virus early-late promoter, H6, followed by a unique polylinker sequence, translational initiation and termination signals, and an early transcription termination signal. These plasmids, pHES1 through 4, allowed for rapid single-step cloning and expression of any open reading frame when recombined in vivo with vP293 and scored for growth on MRC-5 cells.

A search for additional markers to further poxvirus genetics led McClain (23) to study the *u* mutants of Gemmell and Fenner (11). These studies (23) provided the first description of host range mutants of poxviruses. These initial observations were quite helpful in the early studies of poxvirus genetics (8, 24, 35). The host range mutants were interpreted to be defective in some control function required for virus replication (8). Subsequent genomic analysis of these rabbitpox virus mutants (18, 25) demonstrated extensive terminal deletions (up to 30 kilobases [kb]) of DNA.

Host range mutants of vaccinia virus have also been described (4, 5, 10, 15, 22, 39). Nitrous acid mutagenesis of the Copenhagen strain of vaccinia virus allowed Drillien et al. (4) to isolate a host range mutant defective in replication in most human cells. Genomic analysis of this mutant revealed an extensive deletion of approximately 18 kb toward the left terminus (4). Additional analysis by marker transfer studies mapped the genetic function responsible for host range to a 5.2-kb *Eco*RI fragment (12) and finally to an 855-base-pair open reading frame overlapping the *Hin*dIII M and K fragments (13). With reference to previously published results describing overlapping and unique deletions in the left end of the vaccinia virus genome (30), this host range gene is located between 24 and 25.2 kb from the left end of the vaccinia virus L-variant genome. This host range gene, transcribed leftward from *Hin*dIII K into *Hin*dIII M, is described herein as the K1L gene according to the nomenclature recommended by Rosel et al. (33).

We have previously described spontaneous and engineered deletions in the left end of the WR strain of vaccinia virus (27, 30). None of these deletions extended rightward beyond the unique *Bgl*II site in *Hin*dIII-M located 24.1 kb from the left terminus of the L-variant genome (30). None of these deletion mutants demonstrated host range restriction on human cells, consistent with the mapping of the K1L host range gene.

In this communication we report the generation of vP293, a vaccinia virus in which 21.7 kb of DNA, including the K1L gene, was deleted. This virus was defective for growth on the human cell line MRC-5. Further, we report the development of a simple selection system for cloning and expressing open reading frames in a vaccinia virus recombinant based on manipulation of the host range function.

MATERIALS AND METHODS

Cells and virus. The WR strain of vaccinia virus was utilized. Its origin and conditions of cultivation have been previously described (27). Primary chicken embryo fibroblasts (CEF), monkey cell lines (BSC40 and Vero), and the human cell line MRC-5 were cultivated in Eagle minimal essential medium (MEM) containing 5% (BSC40 and Vero cells) or 10% (MRC-5 and CEF cells) fetal bovine serum.

Cloning reagents. Plasmids were constructed, screened, and grown by standard procedures (21, 31, 32). Synthetic *Sma*I linkers were obtained from Collaborative Research, Bedford, Mass. Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; New England BioLabs, Beverly, Mass.; and International Biotechnologies, Inc., New Haven, Conn. The Klenow fragment of *Escherichia coli* DNA polymerase was obtained from Boehringer Mannheim, and phage T4 DNA ligase was obtained from New England BioLabs. The reagents were used as specified by the various suppliers.

Cloning of the neomycin phosphotransferase gene under the control of a vaccinia virus promoter. A fragment containing the gene for neomycin phosphotransferase from transposon Tn5 (1) was isolated from pSV2-neo (37) (ATCC 37149) and put under the control of an early vaccinia virus promoter (designated here as Pi). The Pi promoter had been localized by analysis of early vaccinia virus transcription to a *Sau*3A subclone of the *Ava*I H (*Xho*I G) fragment of the L-variant WR vaccinia virus strain (A. Piccini, unpublished data). This promoter element has been shown to express foreign genes in vaccinia virus recombinants at early times after infection (42). The map location of the promoter is approximately 1.1

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kb from the left end of the *Aval* H fragment (approximately 18.5 kb from the left end of the vaccinia virus genome) and about 9.1 kb left of the *Hind*III C-N fragment junction. More precisely, the Pi promoter DNA sequence corresponds to the region immediately upstream from an open reading frame coding for a 5-kilodalton glycine-rich protein recently reported (17). A 1.5-kb *Sma*I ended Pi promoter-*neo* gene cassette containing 0.5 kb of vaccinia virus DNA including the Pi promoter followed by 1 kb of Tn*5* sequences from the *Bgl*II site through the *Sma*I site (1), was used to construct vaccinia virus recombinant vP293. Recombinant virus was selected by the addition of 300 μg of G418 per ml (9, 37).

Transfection conditions. Procedures for in vivo recombination and in situ hybridization of nitrocellulose filters were as previously described (29), with the following modifications. Plasmid DNA was introduced into vaccinia virus-infected cells by electroporation. Subconfluent monolayers of Vero or MRC-5 cells were infected with rescuing virus for 1 h. The cells were harvested with trypsin, washed with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (14), and electroporated in the presence of 25 μg of plasmid DNA in HEPES-buffered saline. Virus-infected cells were electroporated with a Bio-Rad gene pulser equipped with a Bio-Rad gene pulser capacitance extender. The cell suspension (0.8 ml) was placed on ice for 10 min in a Bio-Rad gene pulser cuvette, pulsed at 200 V (capacitance, 960 μF), and placed on ice for another 10 min. The cells were then diluted in 8 ml of MEM–5% fetal bovine serum, plated (4 ml per dish) in 60-mm dishes containing corresponding Vero or MRC-5 cell monolayers, and incubated at 37°C overnight. At 24 h postinfection the virus was harvested by three freeze-thaw cycles and plated to screen for recombinants.

β-Galactosidase screening. Cell monolayers were infected with virus diluted in Eagle MEM–2% fetal bovine serum. After a 1-h adsorption period, the monolayer was overlaid with Eagle MEM containing 10% newborn calf serum (Flow Laboratories, McLean, Va.), 1% Noble agar, penicillin (1 U/ml), and streptomycin (1 mg/ml) and incubated at 37°C in 5% CO_2. After 3 days the dishes were stained simultaneously with neutral red to visualize colorless plaques and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim Biochemicals) to visualize blue plaques. An agar overlay of equal volume was added over the original overlay. It was prepared by combining equal parts of 2% Noble agar, 0.16% neutral red, and 2× MEM, 4% newborn calf serum, antibiotics, and X-gal (800 μg/ml). X-gal was dissolved in dimethylformamide at 250 mg/ml before it was added to 2× MEM.

Synthetic oligodeoxyribonucleotide. Oligonucleotides were prepared on a Biosearch 8750 or Applied Biosystems 380B DNA synthesizer. All syntheses were performed at the 0.2-μmol scale by using the manufacturers' synthesis cycles for β-cyanoethyl phosphoramidite chemistry. The oligonucleotides were routinely deprotected at 55°C for 12 to 18 h in concentrated NH_4OH and dried in a Speedvac. The DNA was suspended in deionized, distilled H_2O, adjusted to 2.5 M sodium acetate–10 mM $MgCl_2$, and precipitated at −20°C with 2 volumes of ethanol. After centrifugation, the dried oligonucleotides were dissolved in 10 mM Tris hydrochloride (pH 8.0) containing 1 mM EDTA, quantified by A_{260}, and stored at −20°C.

pHES1 was constructed by the following procedure: pMP528HRH was cut with *Xho*I and *Xma*I, and the annealed pair of oligonucleotides HRL1 5'(TCGACCATGGGATCCCCGGGTACCGAGCTCTCGAGTAAATAAATAATTTTTTAT)3' and HRL2 5'(CCGGATAAAAATTATTTATTTACTCGAGAGCTCGGTACCCGGGGATCCCATGG)3' was cloned into this site. pHES2, pHES3, and pHES4 were constructed with similar pairs of annealed oligonucleotides. pHES2 was constructed with the oligonucleotides HRL3 5'(TCGACCATGGGGATCCCCGGGTACCGAGCTCTCGAGTAAATAAATAATTTTTAT)3', and HRL4 5'(CCGGATAAAAATTATTTATTTACTCGAGAGCTCGGTACCCGGGGATCCCCATGG)3', pHES3 was constructed with the oligonucleotides HRL5 5'(TCGACCATGGGGGATCCCCCGGGTACCGAGCTCTCGAGTAAATAAATAATTTTTAT)3' and HRL6 5'(CCGGATAAAAATTATTTATTTACTCGAGAGCTCGGTACCCGGGGATCCCCCATGG)3', and pHES4 was constructed with the oligonucleotides HRL7 5'(TCGAGGATCCCCGGGTACCGAGCTCTAAATAAATAATTTTTAT)3' and HRL8 5'(CCGGATAAAAATTATTTATTTAGAGCTCGGTACCCGGGGATCC)3'.

RESULTS

Construction of the vaccinia virus deletion recombinant vP293. Previous results from our laboratory describing spontaneous and engineered deletions in the left end of the genome of the WR strain of vaccinia virus (27, 30) demonstrated extensive overlapping deletions proximal to the left terminus, but no deletions were obtained extending rightward beyond the unique *Bgl*II site in *Hind*III M located approximately 24.1 kb from the left terminus. These data suggested that essential genetic functions might reside to the right of this locus. This interpretation was consistent with the location of a host range gene described by Drillien's group (4, 12, 13). To extend the previously described overlapping deletions (30) rightward from this locus, we considered engineering deletions by using a selectable marker and screening on a permissive cell to allow for deletion of genes involved in host range. The cloning strategy to achieve this is outlined in Fig. 1. An *Eco*RI-*Sal*I fragment equivalent to the left terminal 3.8 kb of the vaccinia virus genome was isolated from pAG5 (30) and ligated into *Eco*RI-*Sal*I-digested pUC13. The resulting plasmid pMP5 was digested with *Hind*III and *Sal*I and ligated with a 3.8-kb *Hind*III-*Sal*I fragment containing vaccinia virus sequences corresponding to the right end of the vaccinia virus *Hind*III fragment K. The resulting plasmid pMP528 thus contained vaccinia virus sequences at 0 to 3.8 kb and 25.5 to 29.3 kb. The intervening vaccinia virus sequences between the *Sal*I sites at 3.8 and 25.5 kb toward the left end of the genome were effectively deleted. The unique *Sal*I site in pMP528 was changed to a *Sma*I site by the addition of commercially available synthetic linkers producing pMP528L. A 1.5-kb *Sma*I fragment containing the gene for neomycin phosphotransferase from transposon Tn*5* (1) under the control of an early vaccinia virus promoter (designated here as Pi) from the *Ava*I-H region of vaccinia virus was cloned into the *Sma*I site of pMP528L, generating pMP528PiN. pMP528PiN was transfected into primary CEF coinfected with the rescuing vaccinia virus, VTK$^-$79. Recombinant virus was selected and grown on primary CEF in the presence of G418 (9). In the recombinant vaccinia virus vP293, 21.7 kb of vaccinia virus had been deleted, as predicted, and the virus contained the foreign gene encoding Neo^r. The genomic configurations were confirmed by Southern blot hybridization analysis (data not shown). The restriction map of the left terminus of the rescuing virus VTK$^-$79 and of the recombinant virus vP293 expressing the Neo^r gene and selected on primary CEF in the presence of G418 are indicated in Fig. 1B and C.

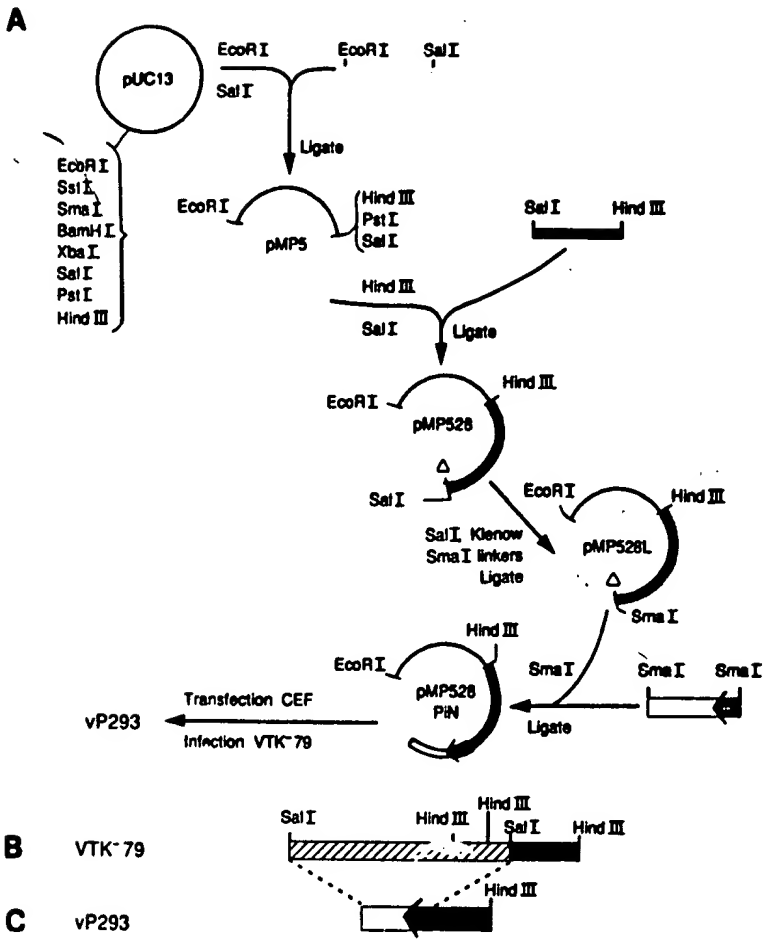

FIG. 1. Construction of plasmid pMP528PiN and generation of vP293. (A) An *Eco*RI-*Sal*I fragment consisting of a 3.8-kb vaccinia virus sequence from pAG5 (30) (left arm, light hatching) was ligated into pUC13 that had been cut with *Eco*RI and *Sal*I, generating pMP5. A *Sal*I-*Hin*dIII fragment consisting of 3.8-kb vaccinia virus sequences from *Hin*dIII-K (right arm, dark hatching) was ligated with pMP5 that had been cut with *Hin*dIII and *Sal*I, generating pMP528. The site of the vaccinia virus deletion between the left and right vaccinia virus arms is indicated by a triangle. pMP528 was cut with *Sal*I and made blunt ended with the Klenow fragment of *E. coli* polymerase; *Sma*I linkers were added, producing pMP528L. pMP528L was cut with *Sma*I and ligated with a *Sma*I-ended cassette containing the neomycin resistance gene (open block) under the control of the early vaccinia virus Pi promoter (dark arrow). The resulting plasmid, pMP528PiN, was transfected into CEF cells infected with VTK⁻79, generating the vaccinia virus recombinant vP293. (B) Map of the left end of VTK⁻79 through *Hin*dIII-K. Only the relevant *Sal*I sites are indicated. The diagonally striped block indicates the vaccinia virus sequences to be deleted. Left and right arms are as indicated above. (C) Map of the left end of vP293 through *Hin*dIII-K. Symbols are as defined above.

In the absence of the antibiotic G418, vP293 produced large plaques on primary CEF and produced plaques well on BSC40 or Vero cells, although vP293 plaques were detectably smaller than the parent VTK⁻79 plaques on Vero cells. Significantly, vP293 gave no measurable replication and failed to form plaques on the human cell line MRC-5. These results are qualitatively consistent with the host range deletion mutant described by Drillien et al. (4).

Reconstitution of vP293 with the host range gene, K1L. Gillard et al. (13) have shown that with their deletion mutant of the Copenhagen strain of vaccinia virus, reconstitution with the host range gene K1L was necessary and sufficient to restore the ability for growth on human cells. This suggested that this host range gene, when reconstituted into the deletion mutant vP293 of the WR strain of vaccinia virus, would also allow the virus to replicate on human cells.

The cloning of the host range gene K1L into plasmid pMP528L and its insertion into vP293 are outlined in Fig. 2. The right vaccinia virus arm of pMP528L (Fig. 1A and 2A) was shortened to eliminate unwanted restriction sites and to facilitate future cloning steps. pMP528L was cut by *Eco*RV-*Hin*dIII, made blunt ended with the Klenow fragment of the *E. coli* polymerase, and self-ligated. The right arm of the resulting plasmid pMP528E was reduced in length to 0.4 kb (Fig. 2A).

An 891-base-pair vaccinia virus *Bgl*II (partial digest)-*Hpa*I fragment containing the entire coding sequence and promoter from the K1L gene (13) was cloned into the polylinker region of pUC8 for the sole purpose of flanking the gene with convenient restriction sites (Fig. 2A). The resulting plasmid, pUC8HR, was digested with *Hin*dIII (partial digest) and *Sma*I to isolate the K1L-containing fragment. The *Hin*dIII

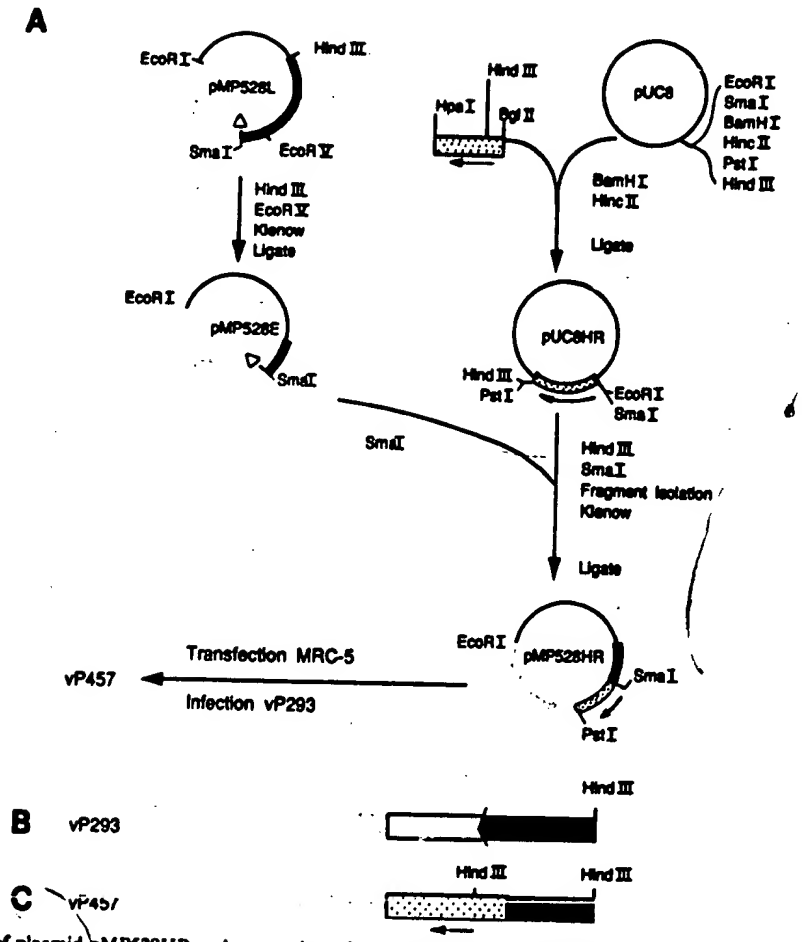

FIG. 2. Construction of plasmid pMP528HR and generation of vaccinia virus recombinant vP457. (A) The right arm of the vaccinia virus deletion plasmid pMP528L (dark hatch) was shortened by digestion with *Hin*dIII and *Eco*RV and then made blunt ended with the Klenow fragment of *E. coli* polymerase. The plasmid was self ligated, generating pMP528E. Regions are indicated by light hatching (left arm), dark hatching (right arm), and triangles (sites of deletion). An *Hpa*I-*Bgl*II fragment containing the K1L gene and its promoter (stippling; direction of gene indicated by arrow) was ligated into pUC8 that had been cut with *Bam*HI and *Hin*cII, generating pUC8HR. A *Hin*dIII (partial digest)-*Sma*I fragment containing the K1L gene was isolated, treated with the Klenow fragment of *E. coli* polymerase, and ligated with pMP528E that had been cut with *Sma*I. The resulting plasmid, pMP528HR, was transfected into MRC-5 cells infected with vP293, generating vaccinia virus recombinant vP457. (B) Map of the left end of vP293 through *Hin*dIII-K. The open block indicates the neomycin resistance gene; the dark arrow indicates the Pi promoter. (C) Map of the left end of vP457 through *Hin*dIII-K. Symbols are as defined above.

end was filled in with the Klenow fragment of the *E. coli* DNA polymerase, and the fragment was cloned into the *Sma*I site of pMP528E. A plasmid, pMP528HR, with the orientation of the K1L gene reading leftward (Fig. 2A) was isolated by standard procedures. pMP528HR contains the host range gene reintroduced into the 21.7-kb deletion in its native right to left orientation with respect to flanking vaccinia virus arms.

The donor plasmid pMP528HR was transfected into either Vero or MRC-5 cells, each coinfected with vP293. Progeny was harvested after an overnight infection and plated on either Vero or MRC-5 cells. The numbers of plaques obtained on Vero cells were 10 to 100 times greater than the numbers of plaques obtained on MRC-5 cells. Isolated plaques of uniform size were picked from MRC-5, and both large and small plaques were picked from Vero cell cultures. These plaque isolates were replated on Vero cells, and after 3 days the resulting plaques were lifted onto nitrocellulose filter disks and prepared for in situ hybridization (26). All of the plaques originating from MRC-5 cells and all of the larger plaques, but not the smaller plaques derived from Vero cells, gave positive hybridization signals when probed with a ^{32}P-labeled probe to the K1L coding sequences. This is consistent with restoration of host range functions contained in the K1L coding sequence. An isolate obtained from MRC-5 cells was further purified and designated vP457. In vP457 the K1L gene has been restored and is situated within the deletion in its native orientation, reading from right to left. The K1L sequences have replaced the Pi promoter-neomycin phosphotransferase gene cassette present in vP293 (Fig. 2B and C). Compared with the genome of the L variant vaccinia virus (27, 30), vP457 contains a 291-base-pair deletion to the right of the K1L gene and a 20.2-kb deletion to the left of the K1L gene. The ability of vP293 and vP457 to form plaques on Vero or MRC-5 cells is shown in Fig. 3. Note that the plaquing efficiency of vP457 is approx-

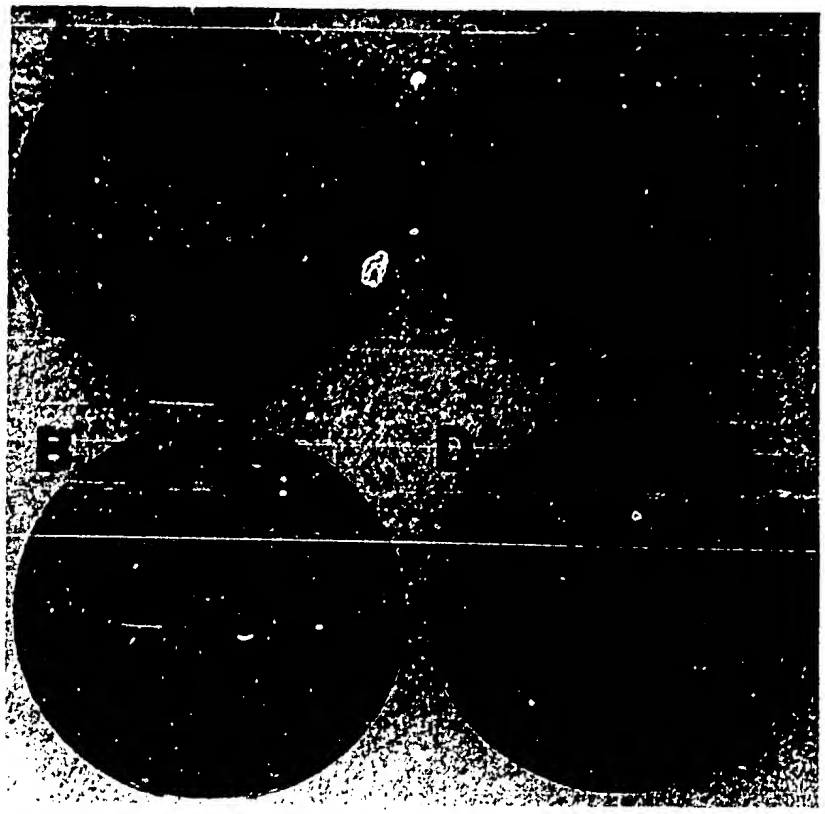

FIG. 3. Plaquing proficiency of vaccinia virus mutants vP293 and vP457 on Vero or MRC-5 cells. Virus was plated on Vero or MRC-5 cells as described in Materials and Methods. Plaques were visualized after 3 days under an agar overlay by staining with neutral red. vP457 was plated on MRC-5 (10^5) (A) or Vero (10^6) (B) cells. vP293 was plated on MRC-5 (10^1) (C) or Vero (10^4) (D) cells.

imately 10 times as high on Vero cells (Fig. 3B, plated at a 10^{-6} dilution) as on MRC-5 cells (Fig. 3A, plated at a 10^{-5} dilution). Under the conditions used here, a similar plaquing differential was typically seen with the wild-type L or S variant viruses (27) plated on Vero and MRC-5 cells (data not shown). No plaques were formed by vP293 on MRC-5 cells (Fig. 3C, plated at a 10^{-1} dilution), even when plated at 1,000 times the concentration used with Vero cells (Fig. 3D, plated at a 10^{-4} dilution).

Construction of plasmids pMP528HRH and pHES1-4. The above results suggested that the conditional lethal phenotype of vP293 could be exploited for constructing donor plasmids into which additional open reading frames could be cloned and expressed. Introduction of these exogenous open reading frames into a plasmid containing the K1L host range gene and recombination into vP293 would yield a simple method for generating vaccinia virus recombinants by virtue of host range restriction. To this end a series of plasmids, pMP528HRH and pHES1 through 4, was constructed.

First, a vaccinia virus promoter was added upstream from the K1L gene in pMP528HR. This early-late promoter was previously identified and localized in *Hin*dIII-H by transcriptional mapping and DNA sequence analysis (A. Piccini and R. Weinberg, unpublished data) and has been utilized to express foreign genes in recombinant fowlpox virus vectors (40, 41). This promoter, H6, maps upstream from the H6 open reading frame and is identical to the sequence published by Rosel et al. (33). Double-stranded DNA corresponding to positions −124 to −1 (with position −102 changed from A to G to remove a potential initiation codon) and followed by *Xho*I, *Kpn*I, and *Sma*I restriction sites was synthesized chemically and cloned into the *Sma*I site of pMP528HR, producing pMP528HRH (Fig. 4A and B). Thus, pMP528HRH contained the H6 promoter upstream from the K1L gene, which was expressed under the control of the K1L endogenous promoter. Both were in a right-to-left orientation with respect to vaccinia virus arms (genome) (Fig. 4). The H6 promoter in pMP528HRH was immediately upstream of unique *Xho*I, *Kpn*I, and *Sma*I restriction sites.

To increase further the utility of the system, plasmids pHES1 through 4 were derived (Fig. 4C through F) from pMP528HRH. In each case pMP528HRH was cut with *Xho*I and *Xma*I, an isoschizomer of *Sma*I, and ligated with the appropriate pair of annealed oligonucleotides. In addition to the elements contained in pMP528HRH, each of plasmids pHES1 through 3 contained a translation initiation codon downstream from the H6 promoter followed by unique multiple restriction sites, translational termination signals, and a specific vaccinia virus early transcription termination signal sequence (44). In each of plasmids pHES1 through 3, the translation initiation codon was in a different reading frame relative to the polylinker region that follows (Fig. 4). Therefore, any DNA sequence that contained an open reading frame could potentially be expressed when cloned into one of these plasmids and recombined into vaccinia virus. A fourth plasmid, pHES4, was also derived from pMP528HRH. This plasmid did not contain a translation initiation codon but did contain unique multiple restriction

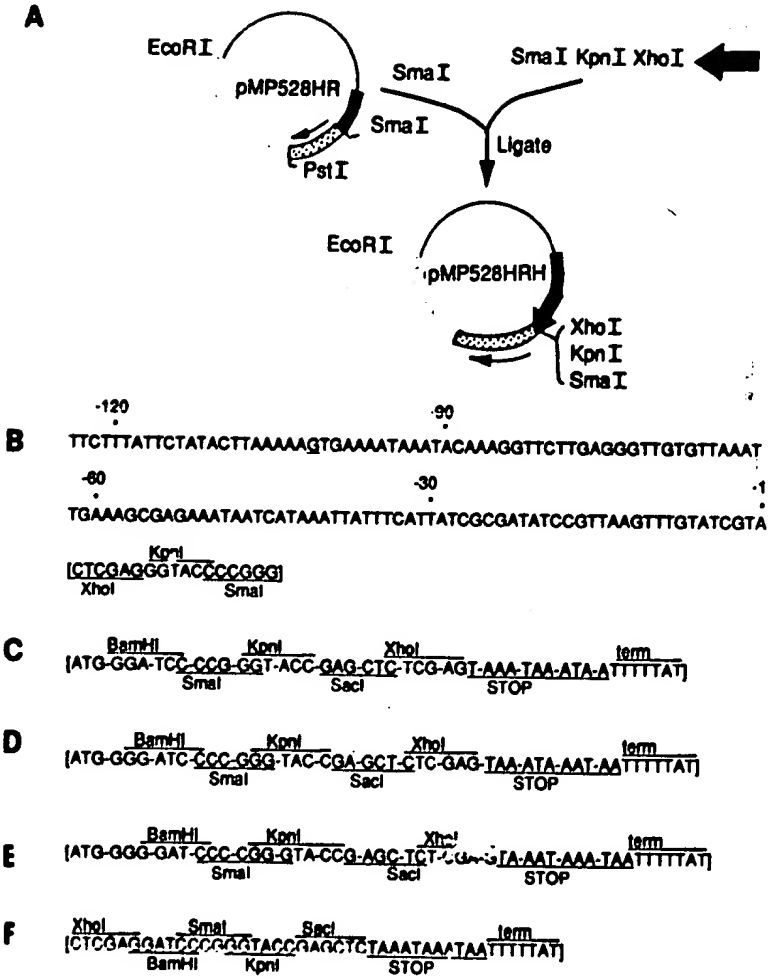

FIG. 4. Construction of plasmids pMP528HRH and pHES1 through 4. (A) pMP528HR (light hatching, left arm; dark hatching, right arm; stippling, K1L gene) was cut with *Sma*I and ligated with a blunt end fragment containing the synthetic H6 promoter positions −124 through −1 (dark arrow), followed by *Xho*I, *Kpn*I, and *Sma*I restriction sites. The resulting plasmid is pMP528HRH. (B) Sequence of synthetic H6 promoter (positions −124 through −1) and downstream restriction sites present in pMP528HRH. Altered base as described in the text at position −102 is underlined. The bracketed sequence is replaced in plasmids pHES1 through 4 (C through F, respectively, below). (C through F) Replacement of bracketed sequence from B (above) in pHES1 through 4. Note that pMP528HRH and pHES4 do not contain an ATG downstream from the H6 promoter, whereas pHES1 through 3 each contain an ATG followed by a frame shift. Restriction sites, stop codons, and early transcriptional termination signals as indicated.

sites, translational termination sequences, and an early transcription termination signal sequence. A DNA sequence that contains an open reading frame and an initiation codon can potentially be expressed when cloned into pHES4 and recombined into vaccinia virus. The pertinent DNA sequence elements, restriction sites, and transcriptional and translational signals of pMP528HRH and pHES1 through 4 are depicted in Fig. 4.

Incorporation of the bacterial *lacZ* gene into vaccinia virus and selection of the recombinant viruses on the basis of host range restriction. To analyze the utility of the pHES1 through 4 and vP293 host range selection system, we chose the *E. coli lacZ* gene encoding β-galactosidase. A *Bam*HI fragment containing codons 8 through the end of the *lacZ* gene was obtained from pMC1871 (36). This *lacZ Bam*HI fragment was cloned into the unique *Bam*HI site of plasmids pHES1 through 4 in the correct orientation. In vivo recombination between the resulting plasmids pHESLZ1, pHESLZ2, pHESLZ3, and pHESLZ4 transfected into Vero cells coinfected with the host range mutant vP293 was performed as described in Materials and Methods. After 24 h, progeny virus was plated on either Vero or MRC-5 cells. When progeny from transfections with pHESLZ1 through 4 were plated on Vero cells and expression of β-galactosidase was assayed in the presence of X-gal, no blue plaques were observed in cells transfected with pHESLZ1, 2, or 4. Significantly, approximately 20% of the plaques generated with plasmid pHESLZ3 gave blue plaques in the presence of X-gal (data not shown). When progeny from transfections with pHESLZ1 through 4 were plated on Vero cells and recombinant viruses were screened by in situ hybridization, 12 to 22% of the plaques gave positive hybridization signals to *lacZ* (Table 1). When analyzed by in situ DNA hybridization (26), every plaque on MRC-5 demonstrated the presence

TABLE 1. Analysis of recombinant *lacZ* vaccinia virus generated with plasmids pHESLZ1 through 4 and VP293 vaccinia virus[a]

Cell line	Stain	Donor plasmid	No. of plaques			% Positive plaques
			Total	Hybridization positive	X-gal positive	
Vero	Neutral red	pHESLZ1	1,056	153		14.5
		pHESLZ2	637	141		22
		pHESLZ3	793	95		12
		pHESLZ4	1,344	269		20
MRC-5	Neutral red	pHESLZ1	60	60		100
		pHESLZ2	56	56		100
		pHESLZ3	ND	ND		
		pHESLZ4	71	71		100
MRC-5	X-gal	pHESLZ1	60		0	0
		pHESLZ2	55		0	0
		pHESLZ3	59		59	100
		pHESLZ4	70		0	0

[a] Donor plasmids pHESLZ1 through 4 were transfected individually into Vero cells infected with vP293 as described in Materials and Methods. After 24 h, progeny were harvested by three freeze-thaw cycles and plated on Vero or MRC-5 cells. Monolayers stained with neutral red were lifted after 3 days onto nitrocellulose filters and prepared for in situ hybridization (26) with a ^{32}P-labeled *lacZ* gene probe. Other MRC-5 dishes were exposed to X-gal, and blue color development was scored after 8 h. ND, Not done.

of the *lacZ* gene (Table 1). β-Galactosidase activity, however, was seen only in plaques on MRC-5 which were derived from pHESLZ3 (Table 1). Only the plasmid construct pHESLZ3 had the *lacZ* gene in frame with the translational initiation codon.

DISCUSSION

Since the initial demonstration of marker rescue in vaccinia virus by Sam and Dumbell (34) and Nakano et al. (26), vaccinia virus has been used extensively for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into live infectious vaccinia virus involves in vivo recombination between vaccinia virus DNA sequences flanking a foreign genetic element in a chimeric donor plasmid and homologous sequences present in the rescuing vaccinia virus (32). Unperturbed, successful recombination occurs at a frequency of approximately 0.1%. Our initial screening strategy involved in situ hybridization of recombinants on replica filters with a radiolabeled probe homologous to the inserted sequences (26, 29).

A number of modifications have been reported to increase the efficiency of recombination itself or to facilitate the identification of recombinants. Among these modifications are the following: use of single-stranded donor DNA (43); identification of recombinants expressing unique enzymatic functions, such as [^{125}I]iododeoxycytidine incorporation into DNA via expression of the herpes simplex virus thymidine kinase (29); use of chromogenic substrates for (co)expression of foreign genes along with β-galactosidase (3, 28); selection for thymidine kinase expression (positive or negative) (20, 29, 30); antibody-based reactions to visualize recombinant plaques (12); use of conditional lethal temperature-sensitive or drug mutants (7, 16); selection of recombinants by using the neomycin resistance gene from Tn*5* and the antibiotic G418 (9); and selection pressures with mycophenolic acid and the *E. coli gpt* gene (2, 6).

We have described in this report a selection system for constructing vaccinia virus recombinants by using a conditional lethal host range mutant. The deletion recombinant mutant vP293 fails to plaque on the human cell line MRC-5. vP293, however, can be readily cultivated and plaqued on nonhuman cells such as Vero, BSC40, and primary CEF cells. The host range function can be restored to vP293 by restoration of the K1L host range gene. Such a recombinant can now plaque on MRC-5. We have designed a number of plasmids, pMP528HRH and pHES1 through 4, which in addition to the K1L host range gene contain another vaccinia virus promoter, unique multicloning restriction sites, appropriate translational start and stop codons, and an early transcription termination signal. Insertion of a foreign open reading frame into these plasmids followed by in vivo recombination will simultaneously restore the host range function (K1L gene) and introduce the foreign open reading frame into the rescuing virus, vP293. The recombinant viruses can now be identified by their ability to plaque on MRC-5 cells. Plasmids pHES1 through 3 contain initiation codons followed by multicloning restriction sites in the three reading frames. Any exogenous open reading frame can be expressed after insertion in one of these plasmids. pHES4, which lacks a translation initiation codon, is designed for expression of exogenous open reading frames that contain their own ATGs.

Advantages of this system include the absence of any non-vaccinia virus exogenous gene in the final recombinant other than the genetic element of interest, no genetic reversion of the virus (since vP293 is a deletion mutant of K1L), and the rapid one-step identification of recombinants. This single-step procedure can also be used for rapid screening of expression of the foreign genetic element, for example, for epitope mapping.

Additional plasmids containing the K1L host range gene have been constructed (unpublished data) where the H6 early-late promoter has been replaced with either a strictly early or a strictly late vaccinia virus promoter. This will allow study of the subtleties of temporal regulation of expression of foreign genetic elements.

It has recently been reported (38) that cowpox virus contains a gene that, when introduced into vaccinia virus, allows vaccinia virus to replicate productively on the normally growth-restrictive CHO cells (5, 15). Other members of the poxvirus family such as fowlpox and swinepox are quite restricted for growth to avians and swine, respectively. These observations suggest that poxviruses have a family of host range functions that determine their relative range of replication competence. The functions of such genes are currently unknown. Information from studying this family of host range functions might be useful in elucidating evolutionary aspects of the poxvirus family.

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LITERATURE CITED

1. Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn*5*. Gene 19: 327–336.
2. Boyle, D. B., and B. E. H. Coupar. 1988. A dominant selectable marker for the construction of recombinant poxviruses. Gene 65:123–128.

3. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: co-expression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5:3403–3409.
4. Drillien, R., F. Koehren, and A. Kirn. 1981. Host range deletion mutant of vaccinia virus defective in human cells. Virology 111:488–499.
5. Drillien, R., D. Spehner, and A. Kirn. 1978. Host range restriction of vaccinia virus in Chinese hamster ovary cells: relationship to inhibition of protein synthesis. J. Virol. 28:843–850.
6. Falkner, F. G., and B. Moss. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. 62:1849–1854.
7. Falkner, Z., P. Srinivasan, R. F. Pacha, and R. C. Condit. 1986. Efficient targeted insertion of an unselected marker into the vaccinia virus genome. Virology 153:97–105.
8. Fenner, F., and J. F. Sambrook. 1966. Conditional lethal mutants of rabbitpox virus. II. Mutants (p) that fail to multiply in PK-2a cells. Virology 29:600–609.
9. Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby. 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol. Cell. Biol. 5:1918–1924.
10. Gargett, J. D., and D. G. Sharp. 1978. Particle heterogeneity in vaccinia virus populations during passage. Virology 88:262–270.
11. Gerrard, A., and F. Fenner. 1960. Genetic studies with mammalian poxviruses. III. White (*u*) mutants of rabbitpox virus. Virology 11:219–235.
12. Gillard, S., D. Spehner, and R. Drillien. 1985. Mapping of a vaccinia host range sequence by insertion into the viral thymidine kinase gene. J. Virol. 53:316–318.
13. Gillard, S., D. Spehner, R. Drillien, and A. Kirn. 1986. Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc. Natl. Acad. Sci. USA 83:5573–5577.
14. Graham, F. L., and A. J. Van der Eb. 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology 54:536–539.
15. Hruby, D. E., D. L. Lynn, R. C. Condit, and J. R. Kates. 1980. Cellular differences in the molecular mechanisms of vaccinia virus host range restriction. J. Gen. Virol. 47:483–488.
16. Kieny, M. P., R. Lathe, R. Drillien, D. Spehner, S. Skory, D. Schmitt, T. Wiktor, H. Koprowski, and J. P. Lecocq. 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature (London) 312:163–166.
17. Kotwal, G. J., and B. Moss. 1988. Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167:525–537.
18. Lake, J. R., and P. D. Cooper. 1980. Deletions of the terminal sequences in the genome of the white pock (μ) and host restricted (p) mutants of rabbitpox virus. J. Gen. Virol. 48: 133–147.
19. Mackett, M., and J. R. Arrand. 1985. Recombinant vaccinia virus induces neutralizing antibodies in rabbits against Epstein-Barr virus membrane antigen gp340. EMBO J. 4:3229–3234.
20. Mackett, M., G. L. Smith, and B. Moss. 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. USA 79:7415–7419.
21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. Mayr, A., V. Hochstein-Mintzel, and H. Stickl. 1975. Abstammung, Eigenschaften, und Verwendung des attenuierten vaccinia-stammes MVA. Infection 3:6–14.
23. McCrae, M. E. 1985. The host range and plaque morphology of rabbitpox virus (RPμ+) and its *u* mutants on chick fibroblast, PK-2a, and L929 cells. Aust. J. Exp. Biol. Med. Sci. 63:31–44.
24. McCrae, M. E., and R. M. Greenland. 1985. Recombination between rabbitpox virus mutants in permissive and nonpermissive cells. Virology 29:516–522.
25. Meyer, R. W., and C. T. Reiha. 1980. The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology 102:119–132.
26. Nakano, E., D. Panicali, and E. Paoletti. 1982. Molecular genetics of vaccinia virus: demonstration of marker rescue. Proc. Natl. Acad. Sci. USA 79:1593–1596.
27. Panicali, D., S. W. Davis, S. R. Mercer, and E. Paoletti. 1981. Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus. J. Virol. 37:1000–1010.
28. Panicali, D., A. Grzelecki, and C. Huang. 1986. Vaccinia virus vectors utilizing the β-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. Gene 47:193–199.
29. Panicali, D., and E. Paoletti. 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA 79:4927–4931.
30. Perkus, M. E., D. Panicali, S. Mercer, and E. Paoletti. 1985. Insertion and deletion mutants of vaccinia virus. Virology 152:285–297.
31. Perkus, M. E., A. Piccini, B. R. Lipinskas, and E. Paoletti. 1985. Recombinant vaccinia virus: Immunization against multiple pathogens. Science 229:981–984.
32. Piccini, A., M. E. Perkus, and E. Paoletti. 1987. Vaccinia virus as an expression vector. Methods Enzymol. 153:545–563.
33. Rosel, J. L., P. L. Earl, J. P. Weir, and B. Moss. 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J. Virol. 60:436–449.
34. Sam, C. K., and K. R. Dumbell. 1981. Expression of poxvirus DNA in co-infected cells and marker rescue of thermosensitive mutants by subgenomic fragments of DNA. Ann. Virol. 132: 135–150.
35. Sambrook, J. F., B. L. Padgett, and J. K. N. Tomkins. 1966. Conditional lethal mutants of rabbitpox virus. I. Isolation of host cell-dependent and temperature-dependent mutants. Virology 29:592–599.
36. Shapira, S. K., J. Chou, F. V. Richaud, and M. J. Casadaban. 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to lacZ gene sequences encoding an enzymatically active carboxyl-terminal portion of β galactosidase. Gene 25:71–82.
37. Southern, P. H., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327–341.
38. Spehner, D., S. Gillard, R. Drillien, and A. Kirn. 1988. A cowpox virus gene required for multiplication in Chinese hamster ovary cells. J. Virol. 62:1297–1304.
39. Tagaya, I., T. Kitamura, and Y. Sano. 1981. A new mutant of dermovaccinia virus. Nature (London) 292:381–382.
40. Taylor, J., R. Weinberg, Y. Kawaoka, R. Webster, and E. Paoletti. 1988. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 6:504–508.
41. Taylor, J., R. Weinberg, B. Languet, P. Desmettre, and E. Paoletti. 1988. Recombinant fowlpox virus inducing protective immunity in non-avian species. Vaccine 6:497–503.
42. Watkinson, M., L. Annelin, C. C. Smith, B. R. Lipinskas, M. E. Perkus, and E. Paoletti. 1987. Protection of guinea pigs from primary and recurrent herpes simplex virus (HSV) type 2 cutaneous disease with vaccinia virus recombinants expressing HSV glycoprotein D. J. Infect. Dis. 155:1188–1197.
43. Wilson, E. M., W. M. Hodges, and D. E. Hruby. 1986. Construction of recombinant vaccinia virus strains using single-stranded DNA insertion vectors. Gene 49:207–213.
44. Yuen, L., and B. Moss. 1987. Oligonucleotide sequences signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. USA 84:6417–6421.

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Host-Range Restriction of Vaccinia Virus E3L–Specific Deletion Mutants

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Abstract. The vaccinia virus (VV) E3L gene product functions as a dsRNA binding protein that is involved in conferring an interferon-resistant phenotype upon the virus. Studies with a vaccinia virus (VV) E3L$^-$ deletion mutant (vP1080) have also demonstrated that the E3L gene product is critical for productive replication on certain cell substrates. While E3L was found to be nonessential for replication in chick embryo fibroblasts (CEFs), virus specifically deleted of E3L was found to be replication deficient in Vero, HeLa, and murine L929 cells. Further, the temporal block in replication appears to differ in these cell systems, as evidenced by the observed timing of protein synthesis inhibition. In Vero cells infected with the VV E3L$^-$ mutant, there was no detectable protein synthesis after 2 hr post-infection, whereas in L929 cells normal protein patterns were observed even at late times post-infection. Expression of a heterologous dsRNA binding protein, the reovirus σ3 protein, by the E3L$^-$ mutant virus restored near wild-type growth characteristics, suggesting the critical nature for regulating dsRNA levels in VV-infected cells.

Key words: Vaccinia virus, host range, E3L ORF, dsRNA binding protein

Vaccinia virus (VV), as the prototypic member of the poxvirus family, represents a unique DNA-containing virus in that it replicates within the cytoplasm of infected cells. As such, the virus encodes most, if not all, of the functions necessary for enabling productive replication to occur in the cytoplasm, including host-range regulatory activities. Previous reports have demonstrated that VV encodes at least two host-range regulatory functions, C7L and K1L (2,3). Extensive analyses of VV mutants deleted of C7L and K1L, alone or in combination, have demonstrated that these functions modify the replication competency of VV on cells derived from tissues of certain species (2,3, unpublished results). More specifically, VV lacking both C7L and K1L retain their ability to efficiently propagate on Vero cells and primary chick embryo fibroblasts (CEFs), but are greatly debilitated in terms of replication competency on cells derived from certain species, including murine, swine, equids, and humans (2,3, unpublished results). Further, C7L and K1L were found to be interchangeable in certain cell systems (porcine and human), while only K1L provided the ability to productively replicate on rabbit kidney (RK-13) cells (3).

Another poxvirus family member, cowpox virus, has been shown to encode an additional host-range function, CP77kDa, which provides

vaccinia virus replication competency on Chinese hamster ovary (CHO) cells (4). The CP77kDa function has been shown to be functionally interchangeable with C7L and/or K1L for enabling replication of VV on human, porcine, and RK-13 cells (3). Interestingly, however, C7L and K1L cannot substitute for CP77kDa in CHO cells, since wild-type VV does not productively replicate on this cell substrate. VV encoding the CP77kDa function, however, has been shown to replicate on CHO cells (4). The precise mechanisms by which these gene products govern the ability of VV to productively replicate on certain cell substrates remains unknown, although they presumably function through interactions with specific cellular polypeptides, as implicated by their host-cell dependence and the occurrence of ankyrin amino acid sequence motifs (5) in at least the K1L and CP77kDa polypeptides. It has been postulated that these host-range proteins may act as anti-apoptotic functions through specific interactions with host cell proteins (6).

The VV E3L open reading frame (ORF) encodes a double-stranded RNA (dsRNA) binding protein (1) with both a nuclear and cytoplasmic localization (7, B.L. Jacobs, unpublished results). Further, E3L has been shown to inhibit activation of the interferon (IFN)-induced protein kinase, PKR, in in vitro assays and has also demonstrated translational stimulatory activity in transient expression assays (8,9). Such properties are consistent with the identity of E3L as the previously reported *s*pecific *k*inase *i*nhibitory *f*actor (SKIF) activity observed in VV-infected cells (10), which downregulates PKR activation and the downstream phosphorylation of eukaryotic initiation factor-2α (eIF-2α) (8,9,11). Phosphorylation of eIF-2α has been correlated with the cessation of protein synthesis initiation (reviewed in 12).

For the reasons cited earlier, SKIF activity has also been implicated in mediating, at least in part, the IFN-resistant phenotype of VV. In this regard, a VV E3L$^-$ mutant (vP1080) was generated and was shown to have an IFN-sensitive phenotype (8,11). Further, lysates derived from cells infected with an E3L$^-$ VV deletion mutant (vP1080) were shown not to contain SKIF activity (8,11).

Generation of the VV E3L$^-$ deletion mutant, vP1080, was accomplished by in vitro recombination on CEFs (13) using the plasmid pMPE3DELBG as donor DNA and wild-type VV (VC-2 isolate; ref. 14) as the recipient virus. Plasmid, pMPE3LDELBG enabled the precise replacement of the E3L ORF with the *E. coli* β-galactosidase gene under the control of a late VV promoter (8). Confirmation of the specific deletion of the E3L ORF and replacement with the β-galactosidase expression cassette was provided by Southern blot analysis of viral DNA, as well as in situ plaque hybridization using a radiolabeled E3L-specific DNA probe (data not shown). Analysis of the vP1080 genome versus the wild-type VV (VC-2 isolate) demonstrated that no other gross genomic alterations occurred in the generation of the E3L$^-$ mutant virus (data not shown).

A putative role of the VV E3L-encoded gene product as a host-range regulatory function was first realized during the derivation of this E3L$^-$ deletion mutant. Attempts at deriving such a mutant were performed on Vero cells without success. Derivation of the VV E3L$^-$ mutant, however, was readily obtainable using primary CEF cell substrates (8). The role of the E3L-encoded gene product as a host-range regulatory function was confirmed when the plaquing efficiency of the VV E3L$^-$ virus (vP1080) was determined on several cell substrates. In these studies, vP1080 displayed near wild-type plaquing efficiencies on primary CEFs and RK-13 cells, but did not produce discernable plaques on Vero cell monolayers (data not shown). The replicative capacity of the E3L$^-$ virus, vP1080, in CEFs, Vero, HeLa, and murine L929 cell systems was further evaluated by assessing output titers following a 24 hr infection period (Table 1). All cell systems infected with wild-type VV displayed increased virus yields of 1–3 $\log_{10}$ after 24 hr. In sharp contrast, in vP1080-infected cell cultures there was no significant increase in virus yield in any cell system tested, except CEFs (Table 1).

Since it has been previously shown that the VV E3L gene product functions as a dsRNA-binding protein, which acts to downregulate PKR activity (8,9,11), it was of interest to determine whether specific E3L deletion resulted in a cell-specific inhibition of protein synthesis fol-

Table 1. Viral yield of vP1080 on various cell substrates

Cells	Virus	HPI	Yield[a]	Yield vP1080/ Yield VC-2
CEF	vP1080	1	5.3	
		24	7.6	0.3
	VC-2	1	5.4	
		24	8.2	
Vero	vP1080	1	5.0	
		24	5.2	0.005
	VC-2	1	5.1	
		24	7.4	
HeLa	vP1080	1	4.8	
		24	4.7	0.0002
	VC-2	1	4.8	
		24	8.4.	
L929	vP1080	1	4.9	
		24	5.3	0.08
	VC-2	1	5.0	
		24	6.4	

Confluent cell monolayers were prepared in 60 mm dishes using seeding densities of 1×10^6 cells (HeLa, Vero, L929) or 2×10^6 cells (CEF). Cell monolayers were infected with vP1080 or wild-type VV (VC-2 isolate) at a multiplicity of infection (moi) of 1 PFU/cell. Inoculations were performed as previously described (14), and virus was harvested at the indicated times and was titrated in duplicate on CEF cells.
[a]Titer expressed as $\log_{10}$ PFU/ml.
HeLa = human cervix epitheloid carcinoma, ATCC CL2; Vero = African green monkey kidney, ATCC CCL 81; L929 = murine fibroblast, ATCC CCL 1; CEF = primary chicken embryo fibroblast cells obtained from 10 to 11-day-old embryos of SPF origin (Select Laboratories, Gainesville, GA); HPI = hours post-infection.

lowing infection with the E3L$^-$ mutant virus, vP1080. Such a phenotype could explain the host-range restriction. To investigate this hypothesis, confluent monolayers of Vero, CEFs, and L929 cells were infected with wild-type VV (VC-2) or E3L$^-$ (vP1080) mutant virus at a multiplicity of infection of 10 PFU/cell and were pulsed with ^{35}S-methionine at various times post-infection (hpi). Proteins were fractionated by NaDodSO$_4$-PAGE and were observed by fluorography, as described previously (8,11).

Lysates derived from wild-type VV (VC-2) infected CEFs, Vero, and L929 cells displayed similar expression kinetics of virus-induced protein synthesis through 24 hpi (Fig. 1A). This was expected, since wild-type VV productively replicates on all three cell substrates. Conversely, in vP1080-infected cells, protein synthesis ap-

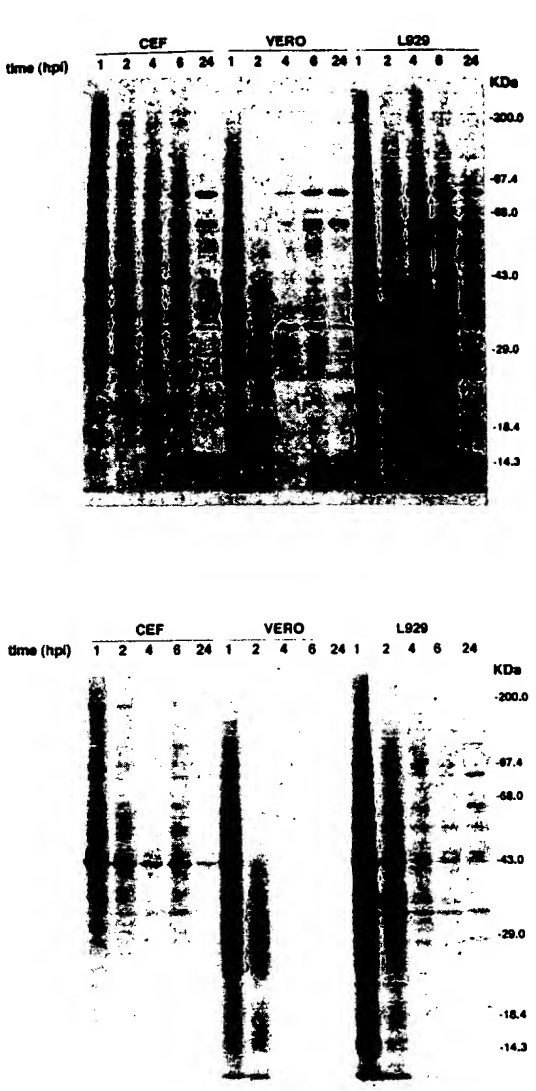

Fig. 1. Expression kinetics of virus-induced protein synthesis. Monolayers of CEF, Vero, and L929 cells were seeded as per Table 1. The cells were infected with wild-type VV (VC-2) (*A*) or E3L-minus vP1080 (*B*) at an moi of 5 PFU/cell. Medium was removed at the indicated times (1, 2, 4, 6, and 24 hr post-infection), and 2 ml methionine-free medium containing 50 μCi [^{35}S]-Translabel was applied to the monolayers. After a 1 hr pulse-labeling period, the cells were scraped into the medium, transferred to a microcentrifuge tube, and the cells were collected by centrifugation. The supernatant was aspirated and the pellet was washed with 1 ml PBS. Pellets were resuspended in 100 μl PBS, and protein was liberated by three cycles of freeze-thaw followed by centrifugation to clarify the lysate. Protein concentrations were determined using the BioRad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA), and equivalent quantities of total protein were fractionated by NaDodSo$_4$-PAGE. The gel was treated for fluorography as previously described (8) and was exposed to Kodak XAR-2 film for visualization.

peared to be reduced to some extent in the cell types tested, including CEFs (Fig. 1B). This was particularly evident at late times post-infection and most likely resulted from protein synthesis inhibition following PKR activation. Vero cells infected with vP1080 displayed highly irregular protein profiles (Fig. 1B). In fact, after 2 hpi there was virtually complete shutdown of virus and host-specific protein synthesis. The apparent host restriction of vP1080 on L929 cells appeared to be temporally distinct from that observed in Vero cells, in that protein profiles in vP1080-infected L929 cells appeared similar to that observed in wild-type VV-infected cells (Fig. 1B). However, no significant levels of measurable infectious virus was produced on L929 cells through this time period (Table 1). It can be inferred from these data that qualitative, as well as quantitative, differences exist between the protein profiles observed in vP1080-infected CEFs, and Vero and L929 cells. This is clearly evidenced when comparing infection of CEFs versus L929 cells, where an anomaly exists between the level of virus-induced protein synthesis and progeny virus production (Table 1; Fig. 1B).

It is intriguing, indeed, that the host-range restriction of the E3L$^-$ VV is temporally distinct between Vero and L929 cells (Fig. 1). This result is reminiscent of previous reports that described the host-range restriction of wild-type VV in CHO (15,16) and Madin-Darby bovine kidney (MDBK) cells (17). Results from these studies demonstrated that virus-induced protein synthesis in CHO-infected cells was defective at intermediate times post-infection, whereas normal virus-induced protein profiles were evident throughout the infection period in MDBK cells. The complexities of poxvirus–host interactions is also exemplified in assessing the nature of the abortive-replication phenotype of avipoxviruses in mammalian cell systems. More specifically, the abortive step in the replication cycle of canarypox virus in many of the mammalian cell systems analyzed to date occurs at early times post-infection (18). In Vero cells, however, the virus displays an abortive-late phenotype, where the abortive step occurs prior to the maturation of immature virus particles (unpublished results).

An initial premise as to the nature of the host-range function provided by E3L involved the dsRNA binding activity of this protein species. To test this hypothesis, we evaluated whether the expression of a heterologous viral dsRNA binding protein, the reovirus σ3 protein, restored a wild-type host-range phenotype to the E3L$^-$ mutant virus, vP1080. Insertion of the σ3-encoding reovirus S4 gene, under the control of the VV early/late H6 promoter (19), into vP1080 was performed as described previously (8). The resultant VV recombinant, vP1112 (8) was compared with wild-type VV (VC-2) and vP1080 for its ability to form plaques on Vero cell monolayers. The results are shown in Fig. 2. As expected, wild-type VV plaqued efficiently, while vP1080 did not plaque on Vero cell monolayers. Significantly, vP1112, which lacks E3L but expresses σ3 protein, was fully capable of forming plaques on Vero cell monolayers (Fig. 2). Previously, vP1112 was shown to overcome the host restriction of an E3L$^-$ VV mutant in HeLa cells,

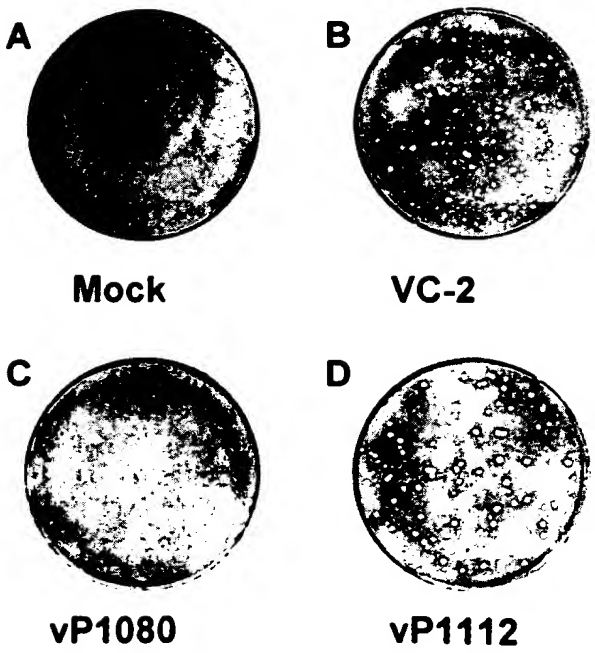

Fig. 2. Ability of wild-type and VV/σ3 virus to form plaques on Vero cells. Virus stocks were diluted to approximately 500 PFU/ml (as determined on CEF cells) and were plated onto monolayers of Vero cells, as described previously (13). Monolayers were stained with crystal violet 3 days after infection to visualize plaques from mock-infected cells (*A*); or cells infected with wild-type, VC-2 (*B*); E3L-minus virus, vP1080 (*C*); or E3L-minus, σ3-expressing virus, vP1112 (*D*).

and rescue of vP1080 by σ3 correlated with its dsRNA binding capacity (8). The suggestion that the dsRNA binding activity mechanistically acts to overcome the host restriction is further supported by results reported by Park et al. (20). In these studies a host cell–derived dsRNA binding protein, TAR RNA binding protein (TRBP), was shown to complement the replication and virus-induced protein synthesis of vP1080 in HeLa cells.

A provocative observation made in the analysis of E3L expression in VV–infected cells was that the gene product is present in the nucleus, as well as the cytoplasm (7; B.L. Jacobs, unpublished results). Since transport of VV-encoded proteins to the nucleus of infected cells is uncommon, it is intriguing to speculate that E3L has an additional role to its function as a dsRNA binding protein. Results cited earlier, as well as recent results, however, do not support this notion, at least with respect to the host-range restriction observed for an E3L$^-$ VV in HeLa cells. In these and other studies (8; Chang and Jacobs, submitted), the ability to complement the E3L$^-$ host-range restriction correlated with the dsRNA binding capacity of either E3L or the heterologous dsRNA binding protein. Further, in vitro mutagenized E3L variants that retain their dsRNA binding capacity but are not transported to the nucleus still complement vaccinia E3L$^-$ virus–induced expression and replication in HeLa cells (Chang and Jacobs, submitted).

Recently, it has been shown that certain viruses encode anti-apoptotic functions to delay the demise of the host cell and to ensure transmission of progeny virus (21–23). Specific to poxviruses, Ink et al. (6) describe results suggesting that the cowpox virus CP77kDa gene product functions as an anti-apoptotic factor. From this, it was inferred that perhaps the VV K1L and C7L gene products function in the same capacity. Utilizing the VV E3L$^-$ mutant, vP1080 (8), it was shown that the VV E3L gene product also functions as an anti-apoptotic factor (24, B.L. Jacobs, unpublished results) by potentially acting through the interferon-induced dsRNA-activated protein kinase, PKR. Such results implicate the existence of a dsRNA-mediated pathway leading to apoptosis. The regulation of dsRNA by VV-encoded functions, therefore, appears critical for suppressing apoptosis in infected cells. In fact, VV appears to have evolved multiple mechanisms that are operative at the transcriptional (i.e., ORF A18R; ref. 25) and post-transcriptional levels (i.e., ORF E3L) to control dsRNA content within the infected cell. Differences in the sensitivities between cell substrates with respect to dsRNA content or in the quantity of synthesized virus-induced dsRNA may serve as the basis for the host-range phenotype illustrated for the VV E3L$^-$ mutant.

References

1. Watson J.C., Chang H., and Jacobs B.L., Virology *185*, 206–216, 1991.
2. Gillard S., Spehner D., Drillien R., and Kirn A., Proc Natl Acad Sci USA *83*, 5573–5577, 1986.
3. Perkus M.E., Goebel S.J., Davis S.W., Johnson G.P., Limbach K., Norton E.K., and Paoletti E., Virology *179*, 276–286, 1990.
4. Spehner D., Gillard S., Drillien R., and Kirn A.J., J Virol *62*, 1297–1304, 1988.
5. Bennett V., J Biol Chem *267*, 8703–8706, 1992.
6. Ink B.S., Gilbert C.S., and Evan G.I., J Virol *69*, 661–668, 1995.
7. Yuwen H., Cox J.H., Yewdell J.R., Bennink J.R., and Moss B., Virology *195*, 732–744, 1993.
8. Beattie E., Denzler K.L., Tartaglia J., Perkus M.E., Paoletti E., and Jacobs B.L., J Virol *69*, 499–505, 1994.
9. Davies M.V., Chang H.-W., Jacobs B.L., and Kaufman R.L., J Virol *67*, 1688–1692, 1993.
10. Whitaker-Dowling P.A. and Youngner J.S. Virology *137*, 171–181, 1984.
11. Beattie E., Paoletti E., and Tartaglia J., Virology *210*, 254–263, 1995.
12. Kaufman R., Curr Opin Biotechnol *5*, 550–557, 1994.
13. Perkus M.E., Kauffman E.B., Taylor J., Mercer S., Smith D., Van Der Hoeven J., and Paoletti E., J Tissue Cult Methods *15*, 72–81, 1993.
14. Tartaglia J., Perkus M.E., Taylor J., Norton, E.K., Audonnet J.-C., Cox W.I., Davis S.W., Van Der Hoeven J., Meignier B., Riviere M., Languet B., and Paoletti E., Virology *188*, 217–232, 1992.
15. Drillien R., Spehner D., and Kirn A., J Virol *28*, 843–850, 1978.
16. Ramsey-Ewing A. and Moss B., Virology *206*, 984-993, 1995.
17. Hruby D.E., Lynn D.L., Condit R.C., and Kates J.R., J Gen Virol *47*, 485–488, 1980.
18. Taylor J., Meignier B., Tartaglia J., Languet B., Van Der Hoeven J., Franchini G., Trimarchi C., and Paoletti E., Vaccine *13*, 539–549, 1995.
19. Perkus M.E., Limbach K., and Paoletti E., J Virol *63*, 3829–3836, 1989.
20. Park H., Davies M.V., Langland J.O., Chang H.-W.,

Nam Y.-S., Tartaglia J., Paoletti E., Jacobs B.L., Kaufman R.J., and Venkatesan S., Proc Natl Acad Sci USA *91*, 4713–4717, 1994.
21. Clem R.J., Fechheimer M., and Miller L.K., Science *254*, 1388–1390, 1991.
22. Shen Y. and Shenk T., Proc Natl Acad Sci USA *91*, 8940–8944, 1994.
23. Tolskaya E.A., Romanova L.I., Kolesnikova M.S., Ivannikova T.A., Smirnova E.A., Raikhlin N.T., and Agol V.I., J Virol *69*, 1181–1189, 1995.
24. Lee S.B. and Esteban M., Virology *193*, 1037–1041, 1993.
25. Bayliss C.D. and Condit R.C., Virology *194*, 254–262, 1993.

2

HOST RANGE RESTRICTED, NON-REPLICATING VACCINIA VIRUS VECTORS AS VACCINE CANDIDATES

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1. INTRODUCTION

The use of a recombinant virus containing a heterologous gene of another microorganism as a live vaccine was suggested more than 10 years ago (Mackett et al., 1982; Panicali and Paoletti, 1982). Vaccinia virus was considered for such a purpose because of its success as a smallpox vaccine and ease and economy of production, distribution and administration (Fenner et al., 1988). The extensive experimental use of recombinant vaccinia viruses was facilitated by the construction of plasmid transfer vectors containing a vaccinia virus promoter, one or more convenient restriction endonuclease sites for inserting a foreign gene, flanking DNA sequences for homologous recombination into a non-essential site of the vaccinia virus genome and for selection and/or screening of recombinant viruses (Chakrabarti et al., 1985; Mackett et al., 1984). Humoral and cell mediated immune responses to an expressed foreign protein and protection of experimental animals against challenge with the corresponding pathogen were demonstated in a variety of animal model systems (Cox et al., 1992; Moss, 1991).

Initial testing of recombinant vaccinia viruses in humans has been reported. A first generation recombinant vaccinia virus AIDS vaccine was considered to be immunogenic and safe (Cooney et al., 1991). Nevertheless, the occurrence of rare adverse reactions to smallpox

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vaccination and the increased susceptibility of immunodeficient individuals has made further attenuation and improved safety a priority for new human vaccines based on vaccinia virus. Attenuation can be achieved by deleting genes that contribute to virulence but are non-essential for replication in tissue culture (Buller et al., 1988; Buller et al., 1985; Tartaglia et al., 1992) or by insertion of lymphokine genes (Flexner et al., 1987; Ramshaw et al., 1987). An alternative approach is to use one of several highly attenuated strains of vaccinia virus that were developed but not extensively used during the smallpox eradication campaign. One of these, known as modified vaccinia virus Ankara (MVA), is avirulent in normal or immunosuppressed animals and elicited no adverse reactions in 120,000 humans, many of whom were at risk for the conventional smallpox vaccine (Hochstein-Mintzel et al., 1972; Mayr and Danner, 1979; Mayr et al., 1975; Mayr et al., 1978; Stickl et al., 1974; Werner et al., 1980).

MVA was generated by over 500 passages of the parental strain in chicken embryo fibroblasts, during which it became severely host restricted and unable to propagate efficiently in mammalian cells. In this respect, MVA resembles Avipoxviruses which are also being developed as safe vaccines (Taylor et al., 1992). Compared to the parental vaccinia virus, MVA contains six major deletions of genomic DNA resulting in the loss of 30,000 base pairs (bp) or 15% of its genetic information (Meyer et al., 1991). The block in replication of MVA in human and other mammalian cells occurs at a step in virion assembly, allowing unimpaired expression of early and late viral or recombinant genes (Sutter & Moss, 1992). Thus, MVA is an efficient as well as a safe vector system. Here, we review examples of the use of recombinant MVA to protectively immunize against influenza virus, simian immunodeficiency virus (SIV), and neoplastic cells in animal model systems.

2. IMMUNIZATION WITH MVA

2.1. Immunization against Influenza Virus

Influenza virus infection of mice provides an experimental model for vaccination against a respiratory disease. Previous studies demonstrated that recombinant vaccinia viruses expressing the influenza virus hemagglutinin gene (*ha*) induced type-specific humoral and cell mediated immune responses and protectively immunized mice against a lethal influenza virus challenge (Andrew et al., 1986; Bennink et al., 1984). Recombinant vaccinia viruses expressing the influenza virus nucleoprotein gene (*np*) induced a less protective but cross-reactive CTL response (Andrew et al., 1986; Yewdell et al., 1985). To evaluate MVA as a candidate vaccine, both the *ha* and *np* regulated by vaccinia virus synthetic strong early/late promoters were inserted into the MVA genome to form MVA-INF$_{ha:np}$ (Sutter et al., 1994). Preliminary experiments verified that the genes were expressed and that the recombinant virus did not cause a spreading infection or discernible cytopathathology in monolayers of mouse L929 cells. Mice inoculated intramuscularly with MVA-INF$_{ha:np}$ developed humoral and CTL immune responses to influenza virus proteins in a dose-dependent manner. A single vaccination with 10^4 or more infectious units of MVA-INF$_{ha:np}$ protected mice against a challenge with 100 times the lethal dose of influenza virus (Table 1; Sutter et al., 1994). Surprisingly, all parameters of immunity including protection were similar or better than those induced by standard intradermal vaccination with equivalent doses of replication competent vaccinia virus strain WR expressing influenza *ha* and *np*. (The lower dose of recombinant virus required for protection than for detectable hemagglutinin inhibition probably reflects different sensitivities of the *in vivo* and *in vitro* assays). Protection was also achieved by nasal vaccination, although higher vaccine doses were required (Table 1).

Table 1. Protection against lethal challenge with influenza virus[1]

Inoculation site	Vaccine	Dose[2]	Increased HI titer[3]	Survivors[4]
Intramuscular	MVA	8	0/8	0/8
	WR	6	0/8	0/8
	WR-INF$_{ha/np}$	4	0/8	5/8
		5	1/8	8/8
		6	4/8	8/8
	MVA-INF$_{ha/np}$	4	0/16	14/16
		5	3/16	16/16
		6	7/8	8/8
Intranasal	MVA	6	0/8	0/8
	MVA-INF$_{ha/np}$	4	0/8	0/8
		5	0/8	0/8
		6	0/8	6/8

[1]data from Sutter et al. (1994) with permission.
[2]log tissue culture infectious dose$_{50}$ or plaque forming unit/animal.
[3]Number of animals with >4 fold increase in hemagglutinin (HI) titer / total animals for each group.
[4]Surviving animals/ total animals challenged for each group; 100 LD$_{50}$ Influenza A/PR/8 challenge delivered to 10-week-old-mice, 4 weeks post vaccination.

2.2. Immunization against SIV

SIV and human immunodeficiency virus (HIV) are closely related viruses with similar genome organizations and CD4 lymphocyte/macrophage tropism. Moreover, SIV causes an immunodeficiency disease in macaques that has many of the features of AIDS. For these reasons, SIV has been used as an HIV surrogate for vaccine studies. Good protection has been obtained by vaccination with live attenuated SIV (Daniel et al., 1992). Varying degrees of protection, perhaps partly due to differences in challenge strains, were observed with recombinant vaccinia viruses alone or combined with other immunogens (Giavedoni et al., 1993; Hu et al., 1992; Israel et al., 1994). MVA could provide a safer alternative to conventional vaccinia virus-based vaccines particularly in populations in which AIDS is prevalent.

A recombinant MVA (MVA-SIV$_{env/gag/pol}$) virus containing the complete envelope and gag-polymerase coding regions of HIV-1 regulated by a strong synthetic vaccinia virus early/late promoter and a moderate natural vaccinia virus early/late promoter, respectively, was constructed (Hirsch et al., 1995). For comparative purposes, the same SIV genes were inserted into the Wyeth (WY) vaccine strain of vaccinia virus to generate WY-SIV$_{env/gag/pol}$. We verified that the SIV genes were expressed by both recombinant viruses in monkey BS-C-1 cells. Twelve juvenile rhesus macaques, divided into four groups, were immunized four times over a period of 28 weeks with MVA-SIV$_{env/gag/pol}$ (n = 4), WY-SIV$_{env/gag/pol}$ (n = 4), MVA control virus (n = 2) or WY control virus (n = 2). After 44 weeks, animals receiving either recombinant virus were also vaccinated with 250 μg of whole SIV, inactivated with psoralen and ultraviolet light (Johnson et al., 1992), in saline. No visible lesions were formed after the intramuscular inoculations of 5 X 10^8 infectius units of MVA or MVA-SIV$_{env/gag/pol}$, whereas typical cutaneous lesions occured after the first intradermal injection of 10^8 infectious units of WY viruses. MVA-SIV$_{env/gag/pol}$ induced a sustained antibody response to both env and gag proteins whereas WY-SIV$_{env/gag/pol}$ induced detectable antibody only to the

Table 2. Responses of vaccinated macaques to SIV challenge

Parameter	MVA or WY controls	MVA-SIVenv/gag/pol	WY-SIVenv/gag/pol
Anamnestic antibody	No	Yes	Yes
PBMC viremia	Persistent in 3 of 4	Transient in 3 of 4	Persistent in 4 of 4
Acute phase			
Antigenemia	3 of 4	None	1 of 4
Plasma viral RNA	High	Severely reduced	Reduced
PBMC viral copies	High	Reduced	Reduced
Lymph nodes			
Morphology	Hyperplastic	Normal	Hyperplastic
In situ	Numerous positive	Negative (3 of 4)	Positive
Virus load	High	Low	Moderate
CD4 lymphocytes	Low (3 of 4)	Low (1 of 4)	Low (4 of 4)
Survivors	1 of 4	4 of 4	1 of 4

envelope protein. *In vitro* neutralizing activity to SIV was transient, peaking after the second recombinant virus administration, and was not enhanced by the inactivated whole SIV.

Four weeks after the final boost, all macaques were challenged by intravenous injection with 50 monkey infectious doses of cell-free, uncloned, homologous SIV (sm/E660) that had been generated in macaque peripheral blood lymphocyte cultures (Goldstein et al., 1994). The results of the SIV challenge are summarized (Table 2; Hirsch et al., 1995). All of the control animals, immunized with non-recombinant MVA or WY strains of vaccinia virus, were infected and three had severe disease requiring them to be sacrificed at 14, 22, and 54 weeks post challenge; the fourth appears healthy with a low virus load and a normal CD4 count. In contrast to the control animals, the macaques that had been vaccinated with recombinant viruses all displayed a rapid anamnestic antibody response to SIV. Although the vaccinated macaques were infected with SIV, virus replication was restricted particularly in three of the four that received MVA-SIV$_{env/gag/pol}$. In the latter, plasma viremia was absent, the viral load in peripheral blood mononuclear cells was reduced, lymph nodes contained 1% or less of the virus from control animals and the architecture was normal, the CD4 counts were maintained, and the animals are still healthy after 62 weeks. The fourth MVA-SIV$_{env/gag/pol}$ immunized macaque has lymphadenopathy and a low CD4 count. The group vaccinated with WY-SIV$_{env/gag/pol}$ was less well protected than the group vaccinated with MVA-SIV$_{env/gag/pol}$: three of the four macaques had to be sacrificed between 51 and 58 weeks because of secondary infections and the fourth has lymphadenopathy and a low CD4 count. At this time, we cannot determine whether inherent genetic differences between the vectors accounted for the better immunity induced by recombinant MVA compared to recombinant WY or whether differences in the dose or route of inoculation were important. Further vaccine trials with MVA-SIV$_{env/gag/pol}$ are in progress.

2.3. Immunization against Neoplastic Cells

Tumor-associated antigens, that are recognized by CD8$^+$ CTL, are potential targets for cancer immunotherapy. To evaluate MVA as a vector for tumor antigens, we used a murine model system that was previously tested with recombinant vaccinia and fowlpox viruses (Bronte et al., 1995; Wang et al., 1995). The BALB/c colon carcinoma cell line CT26.WT was stably transfected with the *Escherichia coli lacZ* gene, which encodes β-galactosidase, to generate CT26.CL25. BALB/c mice, immunized intramuscularly with MVA expressing

Table 3. Protection against neoplastic cells

Vaccination intramuscular	Dose[1]	CT26.WT number of metastases[2]	CT26.CL25 number of metastases[2]
None		411	>500
MVA	10^8	>500	>500
MVA-βgal	10^8	>500	0
MVA-βgal	10^6	>500	2

[1]Infectious units.

[2]Pulmonary nodules per lung, average of 5 animals.

the *lacZ* model tumor antigen (MVA-βgal), were protected against intravenous challenge with a lethal number of CT26.CL25 cells (Table 3). Pulmonary metastases and death occurred in control animals vaccinated with MVA-βgal and challenged with CT26.WT or vaccinated with parental MVA and challenged with CT26.CL25 (Table 3). Therefore, protection was specific for virus and cell-lines expressing the model tumor antigen. In treatment experiments using mice bearing 3-day established pulmonary tumors, either prolonged survival or a reduction in the number of metastases was obtained by immunization with MVA-βgal or by adoptive transfer of *in vitro* stimulated splenocytes from normal mice vaccinated with MVA-βgal. Comparative studies suggested that MVA-βgal might be more effective than WR-βgal (a replication-competent vaccinia virus expressing β-galactosdiase) when used for treatment of established tumors.

3. SUMMARY

Three model sytems were used to demonstrate the immunogenicity of highly attenuated and replication-defective recombinant MVA. (1) Intramuscular inoculation of MVA-IN-$F_{ha/np}$ induced humoral and cell-mediated immune responses in mice and protectively immunized them against a lethal respiratory challenge with influenza virus. Intranasal vaccination was also protective, although higher doses were needed. (2) In rhesus macaques, an immunization scheme involving intramuscular injections of MVA-$SIV_{env/gag/pol}$ greatly reduced the severity of disease caused by an SIV challenge. (3) In a murine cancer model, immunization with MVA-βgal prevented the establishment of tumor metastases and even prolonged life in animals with established tumors. These results, together with previous data on the safety of MVA in humans, suggest the potential usefulness of recombinant MVA for prophylactic vaccination and therapeutic treatment of infectious diseases and cancer.

REFERENCES

Andrew, M. E., Coupar, B. E. H., Ada, G. L. and Boyle, D. B., 1986, Cell-mediated immune response to influenza virus antigens expressed by vaccinia virus recombinants, *Microb. Path.* 1:443-452.

Bennink, J. R., Yewdell, J. W., Smith, J. W., Moller, C. and Moss, B., 1984, Recombinant vaccinia virus primes and stimulates influenza virus HA-specific CTL, *Nature* 311:578-579.

Bronte, V., Tsung, K., Rao, J. B., Chen, P. W., Wang, M., Rosenberg, S. A. and Restifo, N. P., 1995, IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases, *J. Immunol.* 154:5282-5292.

Buller, R. M., Chakrabarti, S., Cooper, J. A., Twardzik, D. R. and Moss, B., 1988, Deletion of the vaccinia virus growth factor gene reduces virus virulence, *J. Virol.* 62:866-877.

Buller, R. M. L., Smith, G. L., Cremer, K., Notkins, A. L. and Moss, B., 1985, Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype, *Nature* 317:813-815.

Chakrabarti, S., Brechling, K. and Moss, B., 1985, Vaccinia virus expression vector: Coexpression of β-galactosidase provides visual screening of recombinant virus plaques, *Mol. Cell. Biol.* 5:3403-3409.

Cooney, E. L., Collier, A. C., Greenberg, P. D., Coombs, R. W., Zarling, J., Arditti, D. E., Hoffman, M. C., Hu, S. L. and Corey, L., 1991, Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein, *Lancet* 337: 567-572.

Cox, W. I., Tartaglia, J. and Paoletti, E., 1992, Poxvirus recombinants as live vaccines, *Recombinant poxviruses*, (Binns, M. M. and Smith, G. L., eds.), 123-162, CRC Press, Boca Raton.

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K. and Desrosiers, R. C., 1992, Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene, *Science* 258:1938-1941.

Fenner, F., Henderson, D. A., Arita, I., Jezek, Z. and Ladnyi, I. D., 1988, *Smallpox and its eradication*, World Health Organization, Geneva.

Flexner, C., Hugin, A. and Moss, B., 1987, Prevention of vaccinia virus infection in immunodeficient nude mice by vector-directed IL-2 expression, *Nature* 330:259-262.

Giavedoni, L. D., Planelles, V., Haigwood, N. L., Ahmad, S., Kluge, J. D., Marthas, M. L., Gardner, M. B., Luciw, P. A. and Yilma, T. D., 1993, Immune response of rhesus macaques to recombinant simian immunodeficiency virus-gp130 does not protect from challenge infection, *J. Virol.* 67:577-583.

Goldstein, S., Elkins, W. R., London, W. T., Hahn, A., Gocken, R., Martin, J. E. and Hirsch, V. M., 1994, Immunization with whole inactivated vaccine protects from infection by SIV grown in human but not macaque cells, *J. Med. Primatol.* 23: 75-82.

Hirsch, V. M., Goldstein, S., Chanock, R., Elkins, W. R., Sutter, G., Moss, B., Sisler, J., Lifson, J. and Fuerst, T., 1995, Limited virus replication following SIV challenge of macaques immunized with attenuated MVA vaccinia expressing SIVsm *env* and *gag-pol*, *Vaccines* 95:195-200.

Hochstein-Mintzel, V., Huber, H. C. and Stickl, H., 1972, Virulenz und immunogenität eines modifizierten vaccinia-virus (Stamm MVA), *Z. Immun.-Forsch.* 144:140-145.

Hu, S.-L., Abrams, K., Barber, G. N., Moran, P., Zarling, J. M., Langlois, A. J., Kuller, L., Morton, W. R. and Benveniste, R. E., 1992, Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160, *Science* 255:456-459.

Israel, Z. R., Edmonson, P. F., Maul, D. H., O'Neill, S. P., Mossman, S. P., Thiriart, C., Fabry, L., Van Opstal, O., Bruck, C., Bex, F., Burny, A., Fultz, P. N., Mullins, J. I. and Hoover, E. A., 1994, Incomplete protection, but suppression of virus burden, elicited by subunit simian immunodeficiency virus vaccines, *J. Virol.* 68:1843-1853.

Johnson, P. R., Montefiori, D. C., Goldstein, S., Hamm, T. E., Zhou, J. Y., Kitov, S., Haigwood, N. L., Misher, L., London, W. T., Gerin, J. L., Allison, A., Purcell, R. H., Chanock, R. M. and Hirsch, V. M., 1992, Inactivated whole SIV vaccine in Macaques - evaluation of protective efficacy against challenge with cell-free virus or infected cells, *AIDS Res. Human Retroviruses*, 8:1501-1505.

Mackett, M., Smith, G. L. and Moss, B., 1982, Vaccinia virus: a selectable eukaryotic cloning and expression vector, *Proc. Natl. Acad. Sci. USA*, 79:7415-7419.

Mackett, M., Smith, G. L. and Moss, B., 1984, General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes, *J. Virol.* 49:857-864.

Mayr, A. and Danner, K., 1979, Bedeutung von tierpocken für den menschen nach aufhebung der pflichtimpfung gegen pocken, *Berl. Münch. Tierärztl. Wochenschr.* 92:251-256.

Mayr, A., Hochstein-Mintzel, V. and Stickl, H., 1975, Abstammung, eigenschaften und verwendung des attenuierten vaccinia-stammes MVA, *Infection* 3:6-14.

Mayr, A., Stickl, H., Müller, H. K., Danner, K. and Singer, H., 1978, Pockenimpfstamm MVA: marker, genetische struktur, erfahrungen mit der parenteralen schutzimpfung und verhalten im abwehrgeschwächten organismus, *Zbl. Bakt. Hyg. I.Abt. Orig. B* 167:375-390.

Meyer, H., Sutter, G. and Mayr, A., 1991, Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence, *J. Gen. Virol.* 72:1031-1038.

Moss, B., 1991, Vaccinia virus: a tool for research and vaccine development, *Science* 252:1662-1667.

Panicali, D. and Paoletti, E., 1982, Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus, *Proc. Natl. Acad. Sci. USA* 79:4927-4931.

Ramshaw, A., Andrew, M. E., Phillips, S. M., Boyle, D. B. and Coupar, B. E. H., 1987, Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection, *Nature* 329:545-546.

Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H. C., Schäfer, H. and Holzner, A., 1974, MVA-stufenimpfung gegen pocken. Kleinische erprobung des attenuierten pocken-lebendimpfstoffes, stamm MVA. *Dtsch. Med. Wschr.* 99:2386-2392.

Sutter, G. and Moss, B., 1992, Nonreplicating vaccinia vector efficiently expresses recombinant genes, *Proc. Natl. Acad. Sci. USA* 89:10847-10851.

Sutter, G., Wyatt, L. S., Foley, P. L., Bennink, J. R. and Moss, B., 1994, A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus, *Vaccine* 12:1032-1040.

Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J. C., Cox, W. I., Davis, S. W., Vanderhoeven, J., Meignier, B., Riviere, M., Languet, B. and Paoletti, E., 1992, NYVAC - A highly attenuated strain of vaccinia virus, *Virology* 188:217-232.

Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Breidis, D., Appel, M., Norton, E. and Paoletti, E., 1992, Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins, *Virology* 187:321-328.

Wang, M., Bronte, V., Chen, P. W., Gritz, L., Panicali, D., Rosenberg, S. A. and Restifo, N. P., 1995, Active immunotherapy of cancer with a non-replicating recombinant fowlpox virus encoding a model tumor-associated antigen. *J. Immunol.* 154:4685-4692.

Werner, G. T., Jentsch, U., Metzger, E. and Simon, J., 1980, Studies on poxvirus infection in irradiated animals, *Arch. Virol.* 64:247-256.

Yewdell, J. W., Bennink, J. R., Smith, G. L. and Moss, B., 1985, Influenza A virus nucleoprotein is a major target for cross-reactive anti-influenza virus cytotoxic T lymphocytes, *Proc. Natl. Acad. Sci. USA* 82:1785-1789.

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Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes

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Abstract

The 'Modified Vaccinia Ankara' (MVA) strain is a potential live vaccine vector. The use of the hemagglutinin (*ha*) gene of the MVA strain as an insertion site for foreign genes was evaluated. To identify the molecular basis of the hemagglutinin-negative (HA^-) phenotype of MVA, the *ha* gene and the region around this gene were sequenced. Amino acid (aa) sequence comparisons with functional hemagglutinins of other vaccinia strains predicted a functional polypeptide. The late part of the promoter region of the *ha* gene, however, was deleted, causing the apparent loss of the *ha* gene function. Nevertheless, insertion of foreign DNA into the *ha* gene allowed generation of functional recombinant viruses, indicating that the *ha*-gene region is a suitable insertion site.

Keywords: Recombinant Modified Vaccinia Ankara; Deletion III; *ha* Gene promoter

1. Introduction

Recombinant vaccinia viruses are presently widely used as vectors for gene expression and their potential as live vaccines has been evaluated (Moss, 1991). Highly attenuated vaccinia strains have been developed, such as the CV-1 strain (Kempe et al., 1968), the MVA strain (Mayr et al., 1978), and the LC16m0 strain (Sugimoto and Yamanouchi, 1994). In addition, highly attenuated genetically engineered strains have been described (Tartaglia et al., 1992). The vaccinia MVA strain has a long passage history in chicken embryo fibroblasts, during which six major deletions (termed deletion I–VI), accompanied by a severe restriction in host range, have occurred (Mayr et al., 1978; Meyer et al., 1991). MVA cannot grow in most mammalian cells and is an ideal candidate for a recombinant vaccine vector. Recently, viral recombinants have been described using the site of deletion III to insert a foreign gene into the MVA genome (Sutter and Moss, 1992) and protective immunity in mice to influenza virus of MVA recombinants has been demonstrated (Sutter et al., 1994).

The *ha* locus is a site into which foreign genes have successfully been integrated, e.g. in the vaccinia virus WR strain (Flexner et al., 1987). The *ha* gene is not essential for growth of the virus in mammalian cell culture, but some degree of viral attenuation is associated with the HA^- phenotype in animals (Buller and Palumbo, 1992). The MVA strain lacks HA functions; the gene, however, seemed unaffected by one of the deletions, termed deletion III, that occurred close to the *ha* locus (Meyer et al., 1991). We have now sequenced the region surrounding the *ha* gene that included deletion III and evaluated the *ha* locus as an insertion site for foreign genes.

2. Experimental and discussion

2.1. Structure of the MVA hemagglutinin gene region

To investigate the genetic basis for the apparent lack of expression of the MVA *ha* gene, the region around the *ha* locus including deletion III was sequenced using

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Abbreviations: aa, amino acid(s); bp, base pair(s); *ha* gene, hemagglutinin gene; HA protein, hemagglutinin protein; HA^-, hemagglutinin-negative; kb, kilo base(s) or 1000 bp; MVA, Modified Vaccinia Ankara; nt, nucleotide(s); ORF, open reading frame.

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purified MVA DNA (plaque purified clone M4) as the template. The complete *ha* ORF, including 74 bp of its promoter, is present in the MVA genome. An aa sequence comparison of the MVA HA with functional HA proteins of the vaccinia strains WR (Smith et al., 1991; Cavallaro and Esposito, 1992), Copenhagen (Goebel et al., 1990), IHD-J (Shida, 1986) and Tiantan (Jin et al., 1989) revealed, that the MVA HA protein had no apparent mutations that would predict its non-functional state (data not shown). Deletion III, however, is located in the *ha* gene upstream region deleting the late promoter including the transcription start site, which is located 141 bp upstream of the translational start site. A comparison of the *ha* gene upstream regions of the MVA and the Copenhagen strains is depicted in Fig. 1. Previous transcriptional characterization of the *ha* gene revealed two promoters, an early and, further upstream, a late promoter (Brown et al., 1991a,1991b). HA activity is only readily detectable late in vaccinia infection, indicating that HA activity is predominantly a function of the late promoter (Brown et al., 1991b). Northern blot analyses of MVA induced late RNAs using typical early and late genes as controls confirmed that late expression is greatly reduced as compared to the vaccinia WR strain (data not shown). The apparent HA^- phenotype of MVA is therefore most probably the result of the truncation of the promoter region. The early promoter and its transcription start site, located 7 bp upstream of the start codon, is not affected by the deletion (Fig. 1). This may explain the low level of hemagglutinin inhibiting antibodies detectable after immunization with MVA (Mahnel and Mayr, 1994). The MVA strain, therefore, is probably greatly reduced in its HA activity, but not fully HA negative.

2.2. Characterization of deletion III

From the sequence analysis the extent and the precise location of deletion III could also be defined. The deletion spans 3501 bp and is located between positions 157 611 and 161 111 relative to the vaccinia Copenhagen sequence (Goebel et al., 1990). As found earlier (Meyer et al., 1991), the A51R ORF is truncated and fused to the 3′ region of the A55R ORF. The ORFs A52R, A53R and A54L are completely deleted. The A55R ORF is deleted except for seven carboxy-terminal amino acids. The coding region of the *ha* gene (ORF A56R) is not affected by the deletion. The intergenic sequence of 49 bp between the A55R and A56R ORFs is unaltered. The residual promoter region of the *ha* gene is 75 bp large and includes the C-terminal part of the A55R ORF.

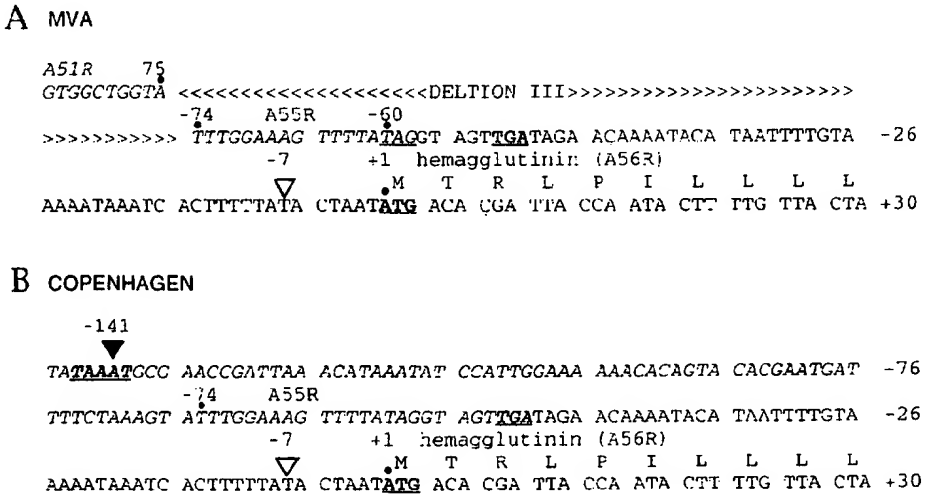

Fig. 1. Comparison of the *ha* gene upstream regions of the vaccinia strains MVA and Copenhagen. The stop codon of ORF A55R and the start codon of ORF A56R are underlined and in bold letters. The A-residue of the start codon of the *ha* gene is defined as position +1. Dots mark the precise location of the positions. The start site for early transcription of the *ha* gene (Brown et al., 1991b is indicated by an open triangle. The full sequence of the MVA *ha* gene and flanking regions were submitted to the EMBL database (accession Mo. X91135). (**A**) In the MVA sequence deletion III (Meyer et al., 1991) is characterized by the fusion of the A-residue of ORF A51R at position −75 (corresponding to position 157 610 in the Copenhagen strain according to the numbering of Goebel et al., 1990) with the T-residue of ORF A55R at position −74 (position 161 112 in the Copenhagen sequence) thus deleting 3501 bp. The early promoter region of the *ha* gene up to position −74 is conserved in MVA and consists of the 49-bp intergenic region between the A55R and A56R ORFs and parts of the A55R ORF. The fusion of the A51R ORF to A55R results in a frame shift; the stop codon of the modified A51R ORF at position 60 is underlined. (**B**) In the wild-type *ha* gene upstream region of the Copenhagen strain the late transcription start site within the conserved TAAAT sequence at position −141 (underlined, bold lettering) is indicated by a black triangle. The promoter region includes the intergenic region and overlaps with the upstream ORF A55R. **Methods**: The DNA sequence of MVA *ha* gene was determined by direct sequencing of viral DNA. Sequencing was performed on an Applied Biosystems Model 373A Sequencer using the cycle sequencing method with dye terminators (PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit #401 628; Perkin Elmer, Inc.) as recommended by the supplier.

2.3. Insertion of foreign genes into the ha locus

The presence of the intact *ha* ORF allowed this gene to be tested as an insertion site. To investigate the behavior of MVA viruses with insertions in the *ha* locus, first the insertion plasmids pHAgpt-oFb and pHAgpt lacZb were constructed (Fig. 2). The latter plasmid was then used in a recombination experiment to insert the *E. coli* genes *lacZ* and *gpt* into the MVA genome. Several *lacZ* and *gpt* positive viruses were selected and plaque purified four times. Identification and purification of plaques could be normally performed. Pure stocks of recombinant virus were obtained after four rounds of plaque purification. Small virus stocks were prepared and used for infections of chicken embryo fibroblasts. After 72 h total DNA was prepared, digested with *Hin*dIII or *Nco*I, separated on a 1% agarose gel and further processed according to a standard Southern blot procedure.

In the *Hin*dIII digests, the expected fragments of about 42 and 5.0 kb were detected (Fig. 3, lanes 1–6 marked '*Hin*dIII'). In the *Nco*I digested genomic DNA, a large fragment of about 9.0 kb, corresponding to the expected size for *lacZ /gpt* insertion into the HA locus, was detectable (lanes 8–13, marked '*Nco*I'). In the wild-type virus controls the expected bands of about 46 kb (lane 7, 'MVA WT') and 3.4 kb (lane 14, 'MVA WT') were found. In addition, the *Nco*I digests confirmed that no wild-type virus was present in the recombinant stocks. Large scale virus preparations revealed that the titers of the recombinants with the *ha* gene insertions were similar to that of the MVA wild-type virus.

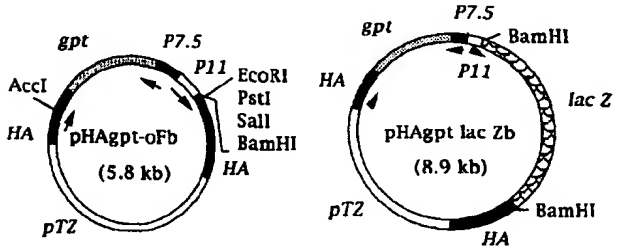

Fig. 2. Schematic representation of the *ha* gene insertion plasmids. *gpt*, *E. coli* xanthine guanine phosphoribosyltransferase gene; *lacZ*, *E. coli* ß-galactosidase gene; P11 (P7.5), promoter of the vaccinia virus gene coding for the 11 kDa (7.5 kDa) polypeptide; pTZ, plasmid sequences of pTZ19R (Pharmacia, Inc.); arrows indicate the orientation of transcription. **Methods**: Construction of the plasmid pHAgptlacZb. To construct this plasmid, first the plasmid pHA was generated by inserting, between the two *Pvu*II sites of pTZ19R (Pharmacia, Inc.), the 1.5-kb *Hin*cII fragment spanning the vaccinia WR hemagglutinin gene. By inserting the 1.7-kb *Hpa*I-*Dra*I fragment (this fragment enharbors the P7.5-*gpt* gene cassette and the P11 promoter including adjacent unique cloning sites and was prepared from plasmid pTKgpt-F1s; Falkner and Moss, 1988) into the unique *Nru*I site of pHA, the orientational isomeric plasmids pHAgpt-oFa and pHAgpt-oFb were obtained (Langmann, 1991). Insertion of the *lacZ* gene, (obtained as a BamHI fragment from plasmid pSC11; Chakrabarti et al., 1985) into pHAgpt-oFb resulted in the plasmid pHAgpt lacZb.

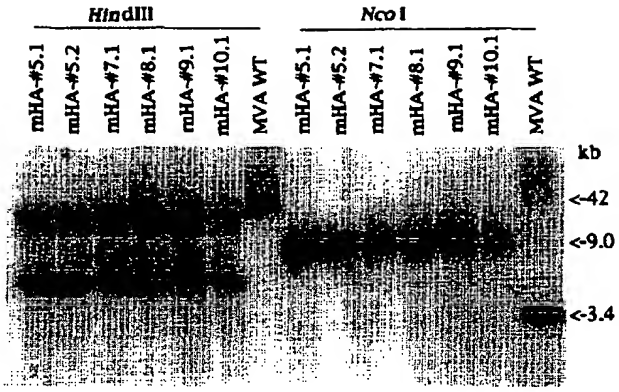

Fig. 3. Southern blot analysis of MVA vaccinia recombinants. Genomic *Hin*dIII and *Nco*I DNA fragments of MVA isolates are shown in the indicated lanes; arrows and numbers at the right side indicate the size of the bands in kb. **Methods**: Construction of the recombinants. Standard vaccinia recombination protocols were used (Mackett et al., 1985). The *lacZ* screening and *gpt* selection procedures have been described previously (Chakrabarti et al., 1985; Falkner and Moss, 1988). For the Southern blots, the genomic *Hin*dIII and *Nco*I fragments were separated by agarose gel electrophoresis, blotted and hybridized to a *ha* gene specific probe (the plasmid pHA).

Although the *ha* locus had previously been shown to be a potential integration site in other vaccinia strains, the special properties of MVA such as extreme attenuation, its apparent HA$^-$ phenotype and highly restricted host range made the evaluation of the *ha* gene as an insertion site necessary. In contrast to insertion of foreign genes into the MVA thymidine kinase gene, which due to further attenuation, is possible only if tk gene activity is restored (Scheiflinger et al., 1996), the MVA *ha* gene is a functional insertion site.

3. Conclusions

The vaccinia virus vaccine strain MVA encodes a *ha* gene with an intact open reading frame, but with a truncated promoter, which is the probable cause of the apparent HA$^-$ phenotype. The early part of the promoter is intact, explaining low residual levels of HA activity.

The MVA strain has lost 3501 bp in the upstream *ha* gene region (deletion III) as compared to the corresponding sequence in the vaccinia strain Copenhagen.

The *ha* gene of the strain MVA is a suitable insertion site for the integration of foreign genes.

Acknowledgement

We thank Prof. A. Mayr for providing the MVA strain, G. Gerstenbauer and M. Jank for expert technical assistance and Dr. C.P. Gibbs for critically reading the manuscript.

References

Brown, C.K., Bloom, D.C. and Moyer, R.W. (1991a) The nature of naturally occurring mutations in the hemagglutinin gene of vaccinia virus and the sequence of immediately adjacent genes. Virus Genes 5, 235–242.

Brown, C.K., Turner, P.C. and Moyer, R.W. (1991b) Molecular characterization of the vaccinia virus hemagglutinin gene. J. Virol. 65, 3598–3606.

Buller, R.M.L. and Palumbo, G.J. (1992) Safety and attenuation of vaccinia virus. In: Binns, M.M. and Smith, G.L. (Eds.), Recombinant Poxviruses. CRC Press, Boca Raton, FL, pp. 235–267.

Cavallaro, K.F. and Esposito, J.J. (1992) Sequences of the raccoon poxvirus hemagglutinin protein. Virology 190, 434–439.

Chakrabarti, S., Brechling, K. and Moss, B. (1985) Vaccinia virus expression vector: coexpression of ß-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5, 3403–3409.

Falkner, F.G. and Moss, B. (1988) *Escherichia coli* gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. 62, 1849–1854.

Flexner, C., Hügin, A. and Moss, B. (1987) Prevention of vaccinia virus infection in immunodeficient mice by vector-directed IL-2 expression. Nature 330, 259–261.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P. and Paoletti, E. (1990) The complete DNA sequence of vaccinia virus. Virology 179, 247–266.

Jin, D., Li, Z., Jin, Q., Hao, Y., Hou, Y. (1989) Vaccinia virus hemagglutinin. A novel member of the immunoglobulin superfamily. J. Exp. Med. 170, 571–576.

Kempe, C.H., Fulginiti, V., Minimitani, M. and Shinfield, H. (1968) Smallpox vaccination of eczema patients with a strain of attenuated live vaccinia (CV1-78). Pediatrics 42, 980–985.

Langmann, T. (1991) Untersuchungen zur Expression von Blutfaktoren unter Anwendung des Vaccinia Virus Systems. Diploma Thesis, Univ. Wien.

Mackett, M., Smith, G.L. and Moss, B. (1985) The construction and characterization of vaccinia virus recombinants expressing foreign genes. In: Glover, D.M. (Ed.), DNA cloning: A practical approach. IRL Press, Oxford, pp. 191–211.

Mahnel, H. and Mayr, A. (1994) Erfahrungen bei der Schutzimpfung gegen Orthopocken von Mensch und Tier mit dem Impfstamm MVA. Berl. Münch. Tierärztl. Wschr. 107, 253–256.

Mayr, A., Stickl, H., Müller, H.K., Danner, K. and Singer, H. (1978) The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism. Zbl. Bakt. Hyg., I. Abt. Orig. B 167, 375–390.

Meyer, H., Sutter, G. and Mayr, A. (1991) Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J. Gen. Virol. 72, 1031–1038.

Moss, B. (1991) Vaccinia virus: a tool for research and vaccine development. Science 252, 1662–1667.

Scheiflinger, F.S., Falkner, F.G. and Dorner, F. (1996) Evaluation of the thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain. Arch. Virol. 141, 663–669.

Shida, H. (1986) Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology 150, 451–462.

Smith, G.L., Chan, Y.S. and Howard, S.T. (1991) Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. J. Gen. Virol. 72, 1349–1376.

Sugimoto, M. and Yamanouchi, K. (1994) Characteristics of an attenuated vaccinia virus strain, LC16m0, and its recombinant virus vaccines. Vaccine 12, 675–681.

Sutter, G. and Moss, B. (1992) Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc. Natl. Acad. Sci. USA 89, 10847–10851.

Sutter, G., Wyatt, L.S., Foley, P.L., Bennink, J.R. and Moss, B. (1994) A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine 12, 1032–1040.

Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.-C., Cox, W.I., Davis, S.W., van der Hoeven, J., Meignier, B., Riviere, M., Languet, B. and Paoletti, E. (1992) NYVAC: a highly attenuated strain of vaccinia virus. Virology 188, 217–232.

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In re Johnson and Farnham

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Headnotes

PATENTS

1. Claims -- Indefinite -- In general (§ 20.551)

Construction of specification and claims -- By prior art (§ 22.20)

Analysis of 35 U.S.C. 112 second paragraph rejection should begin with determination of whether claims satisfy requirements of second paragraph; first inquiry, therefore, is to determine whether claims set out and circumscribe particular area with reasonable degree of precision and particularity; it is here where definiteness of language employed must be analyzed, not in vacuum, but always in light of teachings of prior art and of particular application disclosure as it would be interpreted by one possessing ordinary level of skill in pertinent art.

2. Claims -- Indefinite -- In general (§ 20.551)

Claims -- Specification must support (§ 20.85)

Undue breadth of claims is not indefiniteness.

3. Construction of specification and claims -- By specification and drawings -- In general (§ 22.251)

Claim language must be read in light of specification as it would be interpreted by one of ordinary skill in art.

4. Claims -- Indefinite -- In general (§ 20.551)

Claims -- Specification must support (§ 20.85)

Pleading and practice in Patent Office -- Rejections (§ 54.7)

Specification -- Sufficiency of disclosure (§ 62.7)

Examiner's rejection premised on general ground that claims are "broader than the express limitation disclosed as defining the invention" and specific grounds that "express disclosure is clearly limited to the sigma value recited in claim 1," raises lack of enablement issue properly arising under first not second paragraph of Section 112.

5. Specification -- In general (§ 62.1)

Specification -- Claims as disclosure (§ 62.3)

It is function of specification, not claims, to set forth "practical limits of operation" of invention; one does not look to claims to find out how to practice invention they define, but to specification.

6. Claims -- Specification must support (§ 20.85)

Construction of specification and claims -- In general (§ 22.01)

Specification -- Sufficiency of disclosure (§ 62.7)

Specification as whole must be considered in determining whether scope of enablement provided by specification is commensurate with scope of claims.

7. Construction of specification and claims -- Broad or narrow -- In general (§ 22.101)

Patent grant -- Intent of patent laws (§ 50.15)

Specification -- Sufficiency of disclosure (§ 62.7)

Claims must adequately protect inventors to provide effective incentives; to demand that first to disclose shall limit his claims to what he has found will work or to materials that meet guidelines specified for "preferred" materials in involved process would not serve constitutional purpose of promoting progress in useful arts.

8. Applications for patent -- Continuing (§ 15.3)

Applicants are entitled to benefit of filing date of parent application that discloses invention of application in manner provided by Section 112, paragraph 1.

9. Claims -- Broad or narrow -- In general (§ 20.201)

Estoppel -- Involving interference (§ 35.20)

It is for inventor to decide what bounds of protection he will seek; it is applicant's

right to retreat to otherwise patentable species merely because he erroneously thought he was first with genus when he filed.

10. Specification -- Sufficiency of disclosure (§ 62.7)

Notion that one who fully discloses, and teaches those skilled in art how to make and

use genus and numerous species has failed to disclose and teach those skilled in art how to make and use genus minus two species and has thus failed to satisfy Section 112 first paragraph requirement results from hypertechnical application of legalistic prose relating to that provision of statute.

11. Pleading and practice in Patent Office -- In general (§ 54.1)

Specification -- Sufficiency of disclosure (§ 62.7)

While insufficiency under 35 U.S.C. 112 cannot be cured by citing causes for insufficiency, it is not true that factual context out of which question under Section 112 arises is immaterial; specification having described whole invention necessarily described part remaining after invention of another was excised.

Particular patents -- Polyarylene Polyethers

Johnson and Farnham, Polyarylene Polyethers, rejection of claims 1-9, 64, and 68-72 reversed.

Case History and Disposition:

Appeal from Patent and Trademark Office Board of Appeals.

Application for patent of Robert N. Johnson and Alford G. Farnham, Serial No. 230,091, filed Feb. 28, 1972, continuation-in-part of application Serial No. 295,519, filed July 16, 1963. From decision rejecting claims 1-9, 64, and 68-72, applicants appeal. Reversed; Lane, Judge, dissenting in part with opinion.

Attorneys:

Robert C. Brown and Aldo J. Cozzi, both of New York, N.Y. (James C. Arvantes, New York, N.Y., of counsel) for appellants.

Joseph F. Nakamura (Henry W. Tarring, II, of counsel) for Commissioner of Patents and Trademarks.

Judge:

Before Markey, Chief Judge, and Rich, Baldwin, Lane, and Miller, Associate Judges.

Opinion Text

Opinion By:

Markey, Chief Judge.

This appeal is from the decision of the Patent and Trademark Office (PTO) Board of Appeals affirming the rejection under 35 USC 102 or 103 (the rejection also raises a written description issue under 35 USC 112, first paragraph) of claims 1-9, 64, and 68-70 and the rejection under 35 USC 112, first paragraph (enablement) and second paragraph (indefiniteness), of claims 64 and 68-72 in appellants' application No. 230,091 filed February 28, 1972 (the 1972 application) for "Polyarylene Polyethers." [1] The 1972 application is a continuation-in-part of three earlier applications, the earliest being application No. 295,519 filed July 16, 1963 (the 1963 application). We reverse.

The Invention

The invention is in the field of polymer chemistry and more specifically relates to linear thermoplastic polyarylene polyether polymers composed of recurring units having the general formula

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where O represents an oxygen atom, [2] E represents the residuum of a dihydric phenol [3] compound, and E' represents the residuum of a benzenoid compound having one or more inert electron withdrawing groups [4] in the ortho [5] or para [6] positions to the valence bonds and where both E and E' are bonded to the ether oxygens through aromatic carbon atoms.

Appellants describe a method of synthesizing these polymers by reacting a double alkali metal salt of a dihydric phenol with a dihalobenzenoid compound in the presence of certain solvents under substantially anhydrous reaction conditions.

The 1972 application includes the following disclosure with respect to the electron withdrawing group found in E' and in the E' precursor compound, that is, in the compound which is the predecessor of E' in the above general formula (we have designated paragraphs [A] and [B] and have added emphasis thereto):

> Any electron withdrawing group can be employed as the activator group in these compounds. It should be, of course, inert to the reaction, but otherwise its structure is not critical. Preferred are the strong activating groups such as the sulfone group

> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> bonding two halogen substituted benzenoid nuclei as in the 4,4'-dichlorodiphenyl sulfone and 4,4'-difluorodiphenyl sulfone, although such other strong withdrawing groups hereinafter mentioned can also be used with equal ease.

The more powerful of the electron withdrawing groups give the fastest reactions and hence are preferred. It is further preferred that the ring contain no electron supplying groups on the same benzenoid nucleus as the halogen; however, the presence of other groups on the nucleus or in the residuum of the compound can be tolerated. Preferably, all of the substituents on the benzenoid nucleus are either hydrogen (zero electron withdrawing), or other groups having a positive sigma a1value, as set forth in J.F. Bunnett in Chem. Rev. 49 273 (1951) and Quart. Rev., 12, 1 (1958). See also Taft, Steric Effects in Organic Chemistry, John Wiley & Sons (1956), chapter 13; Chem. Rev., 53, 222; JACS, 74, 3120; and JACS, 75, 4231. [7]

The electron withdrawing group of the dihalobenzenoid compound can function either through the resonance of the aromatic ring, as indicated by those groups having a high sigma a2value, i.e., above about +0.7 or by induction as in perfluoro compounds and like electron sinks.

[A]

Preferably the activating group should have a high sigma a3value, preferably above 1.0, although sufficient activity to promote the reaction is evidenced in those groups having a sigma value above 0.7, although the reaction rate with such a low powered electron withdrawing group may be somewhat low.

The activating group can be basically either of two types:

(a) monovalent groups that activate one or more halogens on the same ring as a nitro group, phenylsulfone, or alkylsulfone, cyano, trifluoromethyl, nitroso, and hetero nitrogen as in pyridine.

(b) divalent group [sic] which can activate displacement of halogens on two different rings, such as the sulfone group

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; the carbonyl group

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; the vinyl group

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; the sulfoxide group

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; the azo group -N=N-; the saturated fluorocarbon groups -CF $_2$CF $_2$-; organic phosphine oxides

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; where R is a hydrocarbon group, and the ethylidene group

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where X can be hydrogen or halogen or which can activate halogens on the same ring such as with difluorobenzoquinone, 1,4- or 1,5- or 1,8- difluoroanthraquinone.

[B]

Those skilled in the art will understand that a plurality of electron withdrawing

groups may be employed if desired, including electron withdrawing groups having a sigma a4value below about +0.7 provided the cumulative sigma a5influence on each of the reactive halogen groups of the halobenzenoid compound is at least about +0.7.

The Disclosure and Prosecution History of the 1963 Application

To understand the written description issue in this appeal, it is necessary to summarize the disclosure and prosecution history of the 1963 application. The 1963 application described (and claimed) in haec verba a genus of polymers as defined by the above general formula. That application stated:

> The high molecular weight polyarylene polyethers of the present invention are the linear thermoplastic reaction products of an alkali metal double salt of a dihydric phenol and a dihalobenzenoid compound. Characteristically, this polymer has a basic structure composed of recurring units having the formula
> -O-E-O-E'-
> wherein E is the residuum of the dihydric phenol and E' is the residuum of the benzenoid compound, both of which are valently bonded to the ether oxygen through aromatic carbon atoms, as hereinafter more fully discussed. Polymers of this type exhibit excellent strength and toughness properties as well as outstanding thermal, oxidative and chemical stability.

The 1963 application then discussed the identity of E and the E precursor compound, that is, the compound which is the predecessor of E in the general formula. It stated:

> The residuum E of the dihydric phenol of these alkali metal salts is not narrowly critical. It can be, for instance, a mononuclear phenylene group as results from hydroquinone and resorcinol, or it may be a di- or polynuclear residuum. Likewise it is possible that the residuum be substituted with other inert nuclear substituents such as halogen, alkyl, alkoxy and like inert substituents.
>
> Such dinuclear phenols can be characterized as having the structure:
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> wherein Ar is an aromatic group and preferably is a phenylene group, Y and Y 1can be the same or different inert substituent groups as alkyl groups having from 1 to 4 carbon atoms, halogen atoms, i.e. fluorine, chlorine, bromine or iodine, or alkoxy radicals having from 1 to 4 carbon atoms, r and z are integers having a value from 0 to 4, inclusive, and R is representative of a bond between aromatic carbon atoms as in dihydroxydiphenyl, or is a divalent radical, including for example, inorganic radicals as
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
> , -O-, -S-, -S-S-, -SO 2-, and divalent organic hydrocarbon radicals such as alkylene, alkylidene, cycloaliphatic, or the halogen, alkyl, aryl or like substituted

alkylene, alkylidene and cycloaliphatic radicals as well as alkalicyclic, alkarylene and aromatic radicals and a ring fused to both Ar group[s].

The application then mentioned by name some fifty specific dihydric dinuclear phenol (bisphenol) compounds which could be the E precursor compound. The application further stated:

> A preferred form of the polyarylene polyethers of this invention are those prepared using the dihydric polynuclear phenols of the following four types, including the derivatives thereof which are substituted with inert substituent groups
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> in which the R group represents hydrogen, lower alkyl, lower aryl and the halogen substituted groups thereof, which can be the same or different.
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
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> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*

Turning to the identity of the E' precursor compound, the application stated:

> Any dihalobenzenoid compound or mixture of dihalobenzenoid compounds

> can be employed in this invention which compound or compounds has the two halogens bonded to benzene rings having an electron withdrawing group in at least one of the positions ortho and para to the halogen group. The dihalobenzenoid compound can be either mononuclear where the halogens are attached to the same benzenoid ring or polynuclear where they are attached to different benzenoid rings, as long as there is the activating electron withdrawing group in the ortho or para position of that benzenoid nucleus.

The 1963 application also included a discussion of the electron withdrawing group that was substantially the same as the paragraphs quoted above from the 1972 application.

The 1963 application contained twenty-six "examples" disclosing in detail the physical and chemical characteristics of fifteen species of polyarylene polyethers. One of the species was the polymer composed of these recurring structural units (which we designate as species [1]): [8]

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Another species disclosed was the polymer composed of these recurring structural units (which we designate as species [2]): [9]
Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.

Appellants' 1963 application became involved in a three-party interference [10] which resulted in an award of priority adverse to appellants from which they did not appeal. [11] "] The sole count of the interference recited species [1].

After their involvement in the interference ended, appellants filed the 1972 application, and they sought broad claims which would at the same time exclude the subject matter of the lost count.

The Claims

Claim 1, now on appeal, is illustrative of the group of claims (claims 1-9, 64, and 68-70) which seek to exclude the subject matter of the lost count and which are involved in the 35 USC 102 or 103 rejection:

> 1. A substantially linear thermoplastic polyarylene polyether composed of recurring units having the general formula:
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> where E is the residuum of a dihydric phenol and E' is the residuum of a benzenoid compound having an inert electron withdrawing group in one or more of the positions ortho and para to the valence bonds having a sigma alvalue above about +0.7, and where both of said residuum [sic, residua] are valently bonded to the ether oxygens through aromatic carbon atoms *with the provisos that E and E' may not both include a divalent sulfone group and may not both include a divalent carbonyl group linking two aromatic nuclei.* [Emphasis added.]

The first "proviso" in claim 1, that "E and E' may not both include a divalent sulfone group," excludes species [1], the species of the lost count. The second "proviso," that "E and E' * * * may not both include a divalent carbonyl group," excludes species [2], which appellants state is "analogous" or "equivalent" to species [1]. [12]

Claims 64 and 71 are illustrative of the group of claims (claims 64 and 68-72) rejected under 35 USC 112, first and second paragraphs:

> 64. A substantially linear thermoplastic polyarylene polyether composed of recurring units having the general formula:
> *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*
>
> where E is the residuum of a dihydric phenol and E' is the residuum of a

benzenoid compound having one or more inert electron withdrawing groups in at least one of the position [sic, positions] ortho and para to the valence bonds having a sigma alvalue *sufficient to activate a halogen atom* and where both of said residuum [sic, residua] are valently bonded to the ether oxygens through aromatic carbon atoms with the provisos that E and E' may not both include a divalent carbonyl group linking two aromatic nuclei. [Emphasis added.]

71. The process for preparing substantially linear polyarylene polyethers which comprises reacting substantially equimolar amounts of an alkali metal double salt of a dihydric phenol with a dihalobenzenoid compound *having halogen atoms activated by an inert electron withdrawing group* in at least one of the positions ortho and para to the halogen atom, under substantially anhydrous conditions and in the liquid phase of an organic solvent having the formula: *Graphic material consisting of a chemical formula or diagram set at this point is not available. See text in hard copy or call BNA PLUS at 1-800-452-7773 or 202-452-4323.*

in which R represents a member of the group consisting of monovalent lower hydrocarbon groups free of aliphatic unsaturation on the alpha carbon atom and, when connected together represents a divalent alkylene group, and Z is an integer from 1 to 2 inclusive. [Emphasis added.]

The Rejections

The sole reference relied upon by the examiner and the board is:
Netherlands 6,408,130 January 18, 1965

Claims 1-9, 64, and 68-70 were rejected under 35 USC 102 or 103 as unpatentable in view of the Netherlands patent, which is a foreign-filed counterpart of appellants' 1963 application.

Before the PTO, appellants conceded that the invention was fully disclosed in the Netherlands patent. However, appellants contended that the claims are entitled to the benefit of the 1963 filing date under 35 USC 120, [13] and therefore the Netherlands patent is not available as a prior art reference.

The examiner and the board were of the view that the claims are not entitled to the 1963 filing date because the presently claimed subject matter is not "described" in the 1963 application as required by the first paragraph of 35 USC 112. [14] As explained by the board:

> The question determinative of the issue at hand is thus whether or not appellants are entitled to the filing date of their parent application Serial No. 295,519, i.e., July 16, 1963. An answer to this question quite obviously depends on what is the invention defined by the instant claims. Is it the same as the one disclosed in [the] parent case or does it differ therefrom in a manner which precludes the instant claims from being afforded the filing date of the parent case?
>
> Under the rationale of the CCPA as set forth in In re Welstead, 59 CCPA 1105, 463 F.2d 1110, 174 USPQ 449 (compare also In re Lukach et al., 58 CCPA 1233, 442 F.2d 967, 169 USPQ 795 , and In re Smith [(I)], 59 CCPA 1025, 458 F.2d 1389, 173 USPQ 679), which we deem controlling, we are constrained to conclude that the present claims are not entitled to the filing date of appellants'

parent case Serial No. 295,519. The claims at issue contain provisos that E and E' may not both include a divalent sulfone group and may not both include a divalent carbonyl group linking two aromatic nuclei. The artificial subgenus thus created in the claims is not described in the parent case and would be new matter if introduced into the parent case. It is thus equally "new matter," i.e., matter new to the present application for

which no antecedent basis exists in the parent case. Consequently, appellants are not entitled to rely on the filing date of their parent case to support a new subgenus for which no basis exists in the parent case. The reason why appellants now limit their claims to exclude those species eliminated by the provisos, i.e., loss in an interference, is manifestly immaterial.

Having reached the conclusion that appellants are not entitled to the filing date of their parent case for the subject matter defined by the present claims which delineate a new subgenus not described in the parent case, it follows that the Netherlands patent is a valid reference which, by appellants' own admission, fully meets the claims. The indicated rejection of claims 1-9, 64 and 68-70 under 35 U.S.C. 102 as unpatentable over the Netherlands patent is thus affirmed. The alternative reliance by the Examiner on Section 103 is inconsequential, Section 102 of the statute being the epitome of Section 103. In re Pearson, (CCPA), 494 F.2d 1399, 181 USPQ 641 .

Claims 64 and 68-72 were rejected under 35 USC 112, first and second paragraphs. In his Answer, the examiner stated that the claims were rejected under §112, first paragraph, for "being broader than the enabling disclosure" and under §112, second paragraph, [15] for being "broader than the express limitations disclosed as defining the invention." The examiner said the "specific deficiencies of the claims and disclosure" are that the expression "to activate a halogen" (claim 64) is "indefinite" because "it does not specify toward what the activation is" and that "[t]he express disclosure is clearly limited to the sigma[a1] value recited in claim 1, for example: see [[A] and [B]]."

In affirming the examiner on these rejections, the board stated:

Further, claims 64 and 68-72 stand finally rejected under 35 U.S.C. 112 as being broader than the enabling disclosure (first paragraph) and broader than the express limitations disclosed as defining the invention (paragraph two).

It is the Examiner's position that "to activate a halogen atom" (claim 64) is indefinite and that the disclosure also is limited to dihalobenzenoid compounds not broadly merely "activated by an inert electron withdrawing group" (claims 68-72) but the activation must have a sigma a2value above about +0.7.

We agree with this rejection. The specification makes it quite clear that a minimum sigma a3activation value of the halogen atoms is required (note especially [[A]]) and an undefined sigma a4value thus lacks the requisite preciseness commensurate with the enablement of the disclosure.

Opinion

I. The Rejections of Claims 64 and 68-72 under §112

Claims 64 and 68-72 were rejected under both the first and second paragraphs of 35

USC 112.

[1]We begin with the rejections under the second paragraph of §112. As stated in In re Moore, 58 CCPA 1042, 1046-1047, 439 F.2d 1232, 1235, 169 USPQ 236, 238 (1971):

> Any analysis in this regard should begin with the determination of whether the claims satisfy the requirements of the second paragraph. * * *
>
> This first inquiry therefore is merely to determine whether the claims do, in fact, set out and circumscribe a particular area with a reasonable degree of precision and particularity. It is here where the definiteness of the language employed must be analyzed -- not in a vacuum, but always in light of the teachings of the prior art and of the particular application disclosure as it would be interpreted by one possessing the ordinary level of skill in the pertinent art. [Footnote omitted.]

The examiner's §112, second paragraph, rejection was premised on the general ground that the claims are "broader than the express limitations disclosed as defining the invention" and on two specific grounds: (a) that the expression "to activate a halogen atom" is "indefinite" because "it does not specify toward what the activation is;" and (b) that "[t]he express disclosure is clearly limited to the sigma[a5] value recited in claim 1, for example: see [[A] and [B]]." The board affirmed and stated: "an undefined sigma a6value thus lacks the requisite *preciseness* * * *." (Emphasis added.)

Ground (a) focuses on the specific phrase "to activate a halogen atom." But the language is found only in claim 64, not in claims 68-72. Claim 68 recites "a dihalobenzenoid compound having halogen atoms activated by an inert electron withdrawing group," and claims 71 and 72 have a similar recitation. (Claims 69 and 70 depend from claim 68.) Those recitations clearly specify "toward what the activation is," as the examiner would require. Ground (a), therefore, lacks merit with respect to claims 68-72.

[2]Product claim 64 [16] defines the complete polymer structure by describing the constituents partially in terms of their functions in the reaction and by their linkage into the end-product polymer. The specification provides further guidance on the meaning of the E' term:

> It is seen also that as used herein, the E' term defined as being the "residuum of the benzenoid compound" refers to the aromatic or benzenoid residue of the compound *after the removal of the halogen atoms on the benzenoid nucleus.* [Emphasis added.]

It is also clear from the specification as a whole, that two keys to the polymerization reaction are inert electron withdrawing groups particularly positioned on the benzenoid nucleus and a cumulative sigma a1value attributable to those withdrawing groups which is sufficient to activate a halogen atom on that nucleus. If the sigma a2value is not sufficient to activate a halogen atom on the benzenoid nucleus, the reaction will not take place and the polymer will not be made. See In re Angstadt, 537 F.2d 498, 190 USPQ 214 (CCPA 1976). The specification adequately details which sigma a3values are sufficient to carry out the reaction, and any person skilled in the art would immediately recognize from the above-quoted portion of the disclosure or the specification as a whole

that the halogen atom mentioned in claim 64 was on the benzenoid nucleus prior to the reaction. It is clear that those skilled in the art would have no trouble ascertaining whether any particular polymer falls within the scope of claim 64. See In re Goffe, 526 F.2d 1393, 188 USPQ 131 (CCPA 1975). The questioned limitation is merely surplusage, since the claim would be definite with or without it. [17]

[3]The point made by the board, that "an undefined sigma a4value" lacks "preciseness," is also unsound. [18] Claim language must be read in light of the specification as it would be interpreted by one of ordinary skill in the art. In re Moore, supra. As pointed out above, those skilled in the art will be able to determine immediately from appellants' detailed specification what level of activation (i.e., sigma a5value) is necessary to practice the invention. Cf. In re Mattison, 509 F.2d 563, 184 USPQ 484 (CCPA 1975). We conclude that the subject matter embraced by claims 64 and 68-72 is definite and that the claims set out and circumscribe a particular area with a reasonable degree of precision and particularity. In re Angstadt, supra; In re Skoll, 523 F.2d 1392, 187 USPQ 481 (CCPA 1975); In re Watson, 517 F.2d 465, 186 USPQ 11 (CCPA 1975); In re Moore, supra. Therefore, the rejection of claims 64 and 68-72 under the *second* paragraph of 35 USC 112 is reversed.

[4]The examiner's general ground and his ground (b) raise a lack of enablement issue properly arising under the *first,* not the second, paragraph of §112. Ground (b) simply supplies the examiner's reasoning in support of the rejection of the claims under §112, first paragraph, as "broader than the enabling disclosure."

As appellants state, the crux of this lack of enablement rejection is that although the specification describes how the halogen atoms bonded to the dihalobenzenoid compound (the E' precursor compound) must be activated in order for polymerization to occur, the claims at issue do not recite a numerical definition of the degree of activation (a minimum sigma a6value) required from the electron withdrawing group. The PTO position is that the claims must recite a minimum sigma a7value in order to conform the scope of the claims to the scope of enablement provided by the specification. The PTO relies on statements [A] and [B] to prove that the scope of enablement

provided by the specification is not commensurate with the scope of the claims.

[5]First, we note that it is the function of the specification, not the claims, to set forth the "practical limits of operation" of an invention. In re Rainer, 49 CCPA 1243, 1248, 305 F.2d 505, 509, 134 USPQ 343, 346 (1962). One does not look to claims to find out how to practice the invention they define, but to the specification. In re Roberts, 470 F.2d 1399, 1403, 176 USPQ 313, 315 (CCPA 1973); In re Fuetterer, 50 CCPA 1453, 319 F.2d 259, 138 USPQ 217 (1963).

[6]Second, we note that the specification *as a whole* must be considered in determining whether the scope of enablement provided by the specification is commensurate with the scope of the claims. In re Moore, supra at 1047, 439 F.2d at 1235, 169 USPQ at 238-39.

The present specification includes broad statements such as: "Any electron withdrawing group can be employed as the activator group in these compounds." The specification also discusses preferred embodiments, alternative embodiments, and the practical limits of operation.

Statement [A] describes preferred embodiments and practical limits of operation. It says that electron withdrawing groups having a high sigma a1value ("preferably above 1.0") are preferred and that the practical limit of operation of the polymerization reaction is reached when the electron withdrawing group has a sigma a2value of 0.7 (at that value the reaction rate "may be somewhat low").

Statement [B] describes an alternative embodiment ("a plurality of electron withdrawing groups") and the practical limit of operation for this embodiment. It states that the cumulative sigma a3influence should be "at least about +0.7."

[7]The PTO would limit appellants to claims reciting a sigma a4value of at least 0.7. This view is improper because it requires the claims to set forth the practical limits of operation for the invention and it effectively ignores the scope of enablement provided by the specification as a whole. As we said in In re Goffe, 542 F.2d 564, 567, 191 USPQ 429, 431 (CCPA 1976):

> [T]o provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for "preferred" materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts. See In re Fuetterer, 50 CCPA 1453, 1462, 319 F.2d 259, 265, 138 USPQ 217, 223 (1963). [Footnote omitted.]

The rejection of claims 64 and 68-72 under the *first* paragraph of 35 USC 112 is reversed.

II. The Rejection of Claims 1-9, 64, and 68-70 Under §102 or §103, Raising Issues Under §112 and §120

[8]We are convinced that the invention recited in claim 1 is "disclosed in the manner provided by the first paragraph of section 112" in the 1963 application and that claim 1 is therefore entitled to the benefit of the 1963 filing date. [19] The only inquiry is whether, after exclusion from the original claims of two species specifically disclosed in the 1963 application, the 1963 disclosure satisfies §112, first paragraph, for the "limited genus" [20] now claimed.

While the board found that "no antecedent basis exists in the parent case" for the "limited genus" in claim 1, we see more than ample basis for claims of such scope. The 1963 disclosure is clearly directed to polymers of the type claimed. Fifty specific choices are mentioned for the E precursor compound, a broad *class* is identified as embracing suitable *choices* for the E' precursor compound, and twenty-six "examples" are disclosed which detail fifteen species of polyarylene polyethers. Only fourteen of those species and twenty-three of the "examples" are within the scope of the claims now on appeal. Two of the many choices for E and E' precursor compounds are deleted from the protection sought, because appellant is *claiming less* than the full scope of his disclosure. But, as we said in In re Wertheim, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976):

> Inventions are constantly made which turn out not to be patentable, and applicants frequently discover during the course of prosecution that only a part of what they invented and originally claimed is patentable.

[9]It is for the inventor to decide what *bounds* of protection he will seek. In re Saunders, 58 CCPA 1316, 1327, 444 F.2d 599, 607, 170 USPQ 213, 220 (1971). To deny appellants the benefit of their grandparent application in this case would, as this court said in Saunders:

> * * * let form triumph over substance, substantially eliminating the right of an applicant to retreat to an otherwise patentable species merely because he erroneously thought he was first with the genus when he filed.

The board cited as "controlling" the decisions of this court in In re Welstead, 59 CCPA 1105, 463 F.2d 1110, 174 USPQ 449 (1972); In re Lukach, 58 CCPA 1233, 442 F.2d 967, 169 USPQ 795 (1971); and In re Smith, 59 CCPA 1025, 458 F.2d 1389, 173 USPQ 679 (1972). Those decisions, because of important factual distinctions, are not controlling.

In Welstead the applicant was attempting to introduce into his claims a new subgenus where "* * * the specification * * * contained neither a description * * * of the [subgenus] * * * nor descriptions of the species thereof amounting in the aggregate to the same thing * * *." Welstead conceded the absence from his disclosure of compounds of the "second type" within the new subgenus. Welstead is thus clearly distinguishable from the present case, in which appellants' grandparent application contains a broad and complete generic disclosure, coupled with extensive examples fully supportive of the limited genus now claimed. Indeed, Welstead might have well been cited by the board in support of a decision contrary to that reached, in view of what this court there implied concerning the possibility that "descriptions of species amounting in the aggregate to the same thing" may satisfy the description requirements of 35 USC 112, paragraph one.

Similarly, in Lukach we noted that "* * * the grandparent application here does not disclose any defined genus of which the presently claimed copolymers are a subgenus." That is not the fact here. Appellants' grandparent application clearly describes the genus and the two special classes of polymer materials excluded therefrom.

In Smith the applicant sought the benefit of his prior application for a broadened generic claim, replacing the claim limitation "at least 12 carbon atoms * * *" with a new limitation calling specifically for 8 to 36 carbon atoms, where there was no disclosure of either the range itself or of a sufficient number of species to establish entitlement to the claimed range. Appellants, in contrast to the applicant in Smith, are narrowing their claims, and the full scope of the limited genus now claimed is supported in appellants' earlier application, generically and by specific examples.

[10]The notion that one who fully discloses, and teaches those skilled in the art how to make and use, a genus and numerous species therewithin, has somehow failed to disclose, and teach those skilled in the art how to make and use, that genus minus two of those species, and has thus failed to satisfy the requirements of §112, first paragraph, appears to result from a hypertechnical application of legalistic prose relating to that provision of the statute. All that happened here is that appellants narrowed their claims to avoid having them read on a lost interference count.

[11]The board indicated that "it is manifestly immaterial" *why* appellants limited their claims. Though it is true that insufficiency under §112 could not be cured by citing the causes for such insufficiency, it is not true that the factual context out of which the question under §112 arises is immaterial. Quite the contrary. Here, as we hold on the facts of this case, the "written description" in the 1963 specification supported the claims

in the absence of the limitation, and that specification, having described the whole, necessarily described the part remaining. The facts of the prosecution are properly presented and relied on, under these circumstances, to indicate that appellants are merely excising the invention of another, to which they are not entitled, and are not creating an "artificial subgenus" or claiming "new matter."

In summary, and for the reasons discussed, the rejections of claims 64 and 68-72 under §112, first and second paragraphs, are reversed; appellants' 1963 disclosure satisfied §112, first paragraph, with respect to claims 1-9, 64, and 68-70 and appellants are, therefore, entitled to the benefit of their 1963 filing date under 35 USC 120. The Netherlands patent is thus rendered unavailable as a prior art reference, and the rejection of the claims under 35 USC 102 or 103 is reversed.

Footnotes

Footnote 1. Claims 10-54 and 65-67 stand allowed. A petition for reconsideration was denied by the board.

Footnote 2. The - O - linkages in the general formula are called ether linkages.

Footnote 3. A dihydric phenol is a type of aromatic organic compound in which two hydroxy (-OH) groups are attached directly to a benzene ring.

Footnote 4. An electron withdrawing group is a substituent which withdraws electrons from the aromatic ring to which it is attached.

Footnote 5. An aromatic ring bearing substituents on adjacent carbon atoms is called ortho substituted.

Footnote 6. An aromatic ring bearing substituents on opposite carbon atoms is called para substituted.

Footnote 7. Appellants' brief specifically refers to one of the publications cited (Chem. Rev., 53, 222 [1953]) and states that its author (Jaffe) defines the sigma a1value as a "special substituent constant" for the "Hammett equation" which is an empirically derived formula intended to show a general quantitative relation between the nature of a given substituent and the reactivity of a side chain. Thus, sigma a2values are based on experimental data and they measure the "activation energy" of a given substituent (electron withdrawing group).

Footnote 8. The -SO_2- linking group in species [1] is called a sulfone group.

Footnote 9. The -CO- linking group in species [2] is called a carbonyl group.

Footnote 10. Interference No. 95,807, declared February 17, 1967.

Footnote 11. Another party did appeal. See Vogel v. Jones, 486 F.2d 1068, 179 USPQ 425 (CCPA 1973).

Footnote 12. The provisos actually exclude more than species [1] and [2]. For example, polymers similar to species [1] and [2] but having substituted ring structures are also excluded.

Footnote 13. §120. Benefit of earlier filing date in the United States.

An application for patent *for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States* by the same inventor shall have the same effect, *as to such invention,* as though filed on the date of the prior application, if filed before the patenting or abandonment of

or termination of proceedings on the first application or on an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application. [Emphasis added.]
Footnote 14.

§112. Specification.

The specification shall contain *a written description of the invention,* and of the manner and process of making and using it, *in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same,* and shall set forth the best mode contemplated by the inventor of carrying out his invention. [Emphasis added.]
Footnote 15.

§112. Specification.

* * *

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Footnote 16.

Claims 68-70 are product-by-process claims.

Footnote 17. We do not speculate on whether or not the claim would be unduly broad if the questioned limitation were removed. But undue breadth is not indefiniteness. In re Borkowski, 57 CCPA 946, 422 F.2d 904, 164 USPQ 642 (1970). This claim is definite either with or without the phrase "to activate a halogen atom."

Footnote 18. In re Merat, 519 F.2d 1390, 186 USPQ 471 (CCPA 1975), cited by the Solicitor, affirmed a §112, second paragraph, rejection because the same word ("normal") was used in the claims in one sense and in the specification in a different sense, thus rendering the claims indefinite. There is nothing akin to the Merat situation here.

Footnote 19. Appellants have not argued the claims separately, thus, claims 2-9, 64, and 68-70 stand or fall with claim 1.

Footnote 20. Appellants refer to the subject matter recited in claim 1 as a "limited genus." The board called it an "artificial subgenus." We use appellants' terminology. Whatever the label, the issue is the same.

Dissenting Opinion Text

Dissent By:

Lane, Judge, dissenting in part,

I would affirm the rejection of claims 64 and 68-72 under §112, paragraphs 1 and 2, because the specification indicates that a

minimum sigma value of +0.7 is an *essential requisite.* These claims fail to recite this requisite, thus fail to define appellants' invention and are broader than the disclosure. I concur in reversing the rejection of claims 1-9.

- End of Case -

Journal of General Virology (1998), **79**, 347–352. Printed in Great Britain

SHORT COMMUNICATION

Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells

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Although desirable for safety reasons, the host range restrictions of modified vaccinia virus Ankara (MVA) make it less applicable for general use. Propagation in primary chicken embryo fibroblasts (CEF) requires particular cell culture experience and has no pre-established record of tissue culture reproducibility. We investigated a variety of established cell lines for productive virus growth and recombinant gene expression. Baby hamster kidney cells (BHK), a well-characterized, easily maintained cell line, supported MVA growth and as proficient expression of the *E. coli lacZ* reporter gene as the highly efficient CEF, whereas other cell lines were non-permissive or allowed only very limited MVA replication. Importantly, no virus production occurred in patient-derived infected primary human cells. These results emphasize the safety and now improved accessibility of MVA for the development of expression vectors and live recombinant vaccines.

The successful worldwide eradication of smallpox via vaccination with live vaccinia virus stimulated research into development of the latter as a highly versatile eukaryotic transient expression vector. Its possible use as a recombinant vaccine for protective immunization against infectious diseases or cancer is particularly attractive (Mackett *et al.*, 1982; Panicali & Paoletti, 1982; for review see Moss, 1996). However, infection with conventional vaccinia virus poses a health risk to both researchers and future patients, particularly immunocompromised individuals. Several attenuated vaccinia virus strains were developed during the smallpox era to reduce possible side effects associated with using live vaccinia virus vaccines (Fenner *et al.*, 1988). In particular, the host range-restricted modified vaccinia virus Ankara (MVA) proved to be extremely attenuated when compared to wild-type vaccinia virus strains (Mayr *et al.*, 1975, 1978; Werner *et al.*, 1980). In clinical trials, MVA was administered without significant side-effects to about 150000 individuals, including many considered at risk for the conventional smallpox vaccination (Stickl *et al.*, 1974; Mahnel & Mayr, 1994). MVA was originally derived from the vaccinia strain Ankara by over 570 serial passages in primary chicken embryo fibroblasts (CEF) severely compromising its capacity to replicate in mammalian cells (Mayr *et al.*, 1975). Further studies revealed that six major deletions had occurred in the DNA of the attenuated virus compared to the parental genome (Meyer *et al.*, 1991). Virus replication is blocked late in morphogenesis in non-permissive cells, but importantly, viral and recombinant protein synthesis is unimpaired at early and late times after infection (Sutter & Moss, 1992). Hence, replication-deficient recombinant MVA was established as an exceptionally safe viral vector (Sutter & Moss, 1995; Moss *et al.*, 1996) and can be used in Germany and the USA under biosafety level 1 conditions (Stellungnahme der Zentralen Kommission für Biologische Sicherheit, AZ 6790-10-14, Berlin, FRG, 5/1997; Moss, 1996). MVA recombinants expressing bacteriophage T7 RNA polymerase genes have been constructed (Wyatt *et al.*, 1995; Sutter *et al.*, 1995) and used successfully as expression systems for reverse genetics of RNA viruses (Collins *et al.*, 1995; Schneider *et al.*, 1997; Baron & Barrett, 1997). When tested in animal model systems recombinant MVA have been shown to be avirulent, yet protective vaccines for immunization against viral diseases and cancer (Sutter *et al.*, 1994; Hirsch *et al.*, 1996; Wyatt *et al.*, 1996; Carroll *et al.*, 1997). Despite, or rather because of the advantages conferred by the severe host range restriction of MVA, its propagation is not trivial, being restricted to primary CEF cells. The establishment and maintenance of CEF cultures requires experience in preparing primary tissue culture and depends on egg material from chicken kept under special pathogen-free conditions. In addition, CEF cultures survive only a few passages and weekly *de novo* preparations are

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Table 1. MVA one-step growth in established cell lines

Cells infected with 10 IU per cell MVA-LZ were harvested after the adsorption period (0 h) or 24 h after infection. Virus titres (T_0, T_{24}) were determined by backtitration on CEF. Virus multiplication is demonstrated by the ratios of the average virus titres determined after 24 h over the titres at the beginning of infection (T_{24}/T_0) in two independent titrations from a single one-step growth experiment. In two additional one-step growth experiments T_{24}/T_0 values of 136 and 250 for CEF, 42 and 56 for BHK, and 4 and 7 for CV-1 were calculated.

Cell origin	Name	T_{24}/T_0
Animal		
Chicken embryo	CEF	316
Baby hamster kidney	BHK	66
Monkey kidney	CV-1	32
Monkey transformed B-cell	MIB	< 1
Human		
Cervix carcinoma	HeLa	5
Melanoma	SK 29 MEL 1	< 1
Transformed kidney	293	4
Embryonic lung	LC 5	< 1
Astrocytoma	85 HG 66	< 1
Glioblastoma	U 138	< 1
T-cell lymphoma	C 8166	< 1
T-cell lymphoma	HUT 78	< 1
Transformed B-cell	SY 9287	< 1

required. Therefore, a cell line that is easily and stably maintained would not only be very useful for experimental propagation of MVA vectors, but also represent a desirable tool for making recombinant MVA vaccines, where ease, low costs, reproducibility and a pre-established safety record of production are essential.

In this study, immortalized and primary human and animal cell lines (Table 1) were investigated for productive growth of a recombinant MVA which encodes the *E. coli lacZ* reporter gene under the control of the vaccinia virus late promoter P11 (MVA-LZ; Sutter & Moss, 1992). In addition, a vaccinia strain Copenhagen recombinant virus encoding the *E. coli lacZ* gene under the control of the early/late promoter P7.5 (VV-LZ; courtesy of Robert Drillien, University of Strasbourg, France) was used. MVA-LZ or VV-LZ were routinely propagated and titred by endpoint dilution in CEF or MA104 cells, respectively, to obtain the $TCID_{50}$. The animal cell cultures investigated were: primary chicken embryo fibroblasts (CEF); baby hamster kidney (BHK; gift from Lothar Schneider, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany); the monkey kidney cell lines MA104 (Rhône Mérieux, Lyon, France) and CV-1 (ATCC CCL 70); and a herpes papiovirus-transformed monkey lymphoblastoid B-cell line, MIB. The human cell lines were: T-cell lymphoma C8166 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP013) and HUT78 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP002); glioblastoma U138MG (Medical Research Council, AIDS Reagent Project, Repository Reference ADP028) and astrocytoma 85HG-66 (Brack-Werner *et al.*, 1992); embryonal lung fibroblasts LC5 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP026; Mellert *et al.*, 1990); cervix carcinoma HeLa (ATCC CCL 2); Epstein–Barr virus-transformed lymphoblastoid B-cell line SY9287 (a gift from Robert Drillien, University of Strasbourg, France); and melanoma SK29MEL1 (a gift from Thomas Wölfel, University of Mainz, Germany). Primary human cells were: fibroblasts (HF) obtained from fresh skin biopsy material; peripheral blood mononuclear cells (PBMC) isolated by Ficoll gradient purification; monocytes (MO) or dendritic cells (DC) separated by anti-CD14 cell rosetting followed by Percoll gradient purification or magnetic sorting (MACS), respectively. All cell cultures were grown in RPMI 1640 supplemented with 10% foetal calf serum (FCS) in a humidified air–5% CO_2 atmosphere. The DC culture medium additionally contained IL4 (400 U/ml) and GM-CSF (50 ng/ml).

To determine low-multiplicity growth profiles, virus multiplication was monitored after infecting cell monolayers and suspension cultures with 0·05 infectious units (IU) MVA-LZ per cell, as described previously (Meyer *et al.*, 1991). Additionally, one-step growth of MVA-LZ was analysed infecting cells at an m.o.i. of 10 IU. For all infection experiments either 10^6 cells in suspension or confluent monolayers from one well of six-well tissue culture plates were used per time point. After virus adsorption for 45 min at 37 °C for low-multiplicity growth or 30 min at 4 °C for the one-step growth experiments, the inoculum was removed. The infected cells were washed three times with RPMI 1640 and incubated with fresh RPMI 1640 medium containing 10% FCS at 37 °C in a 5% CO_2 atmosphere. At multiple time-points post-infection (p.i.) infected cells were harvested and virus was released by freeze–thawing and brief sonication. Serial dilutions of the resulting lysates were plated on confluent CEF monolayers grown in 96-well plates as replicates of eight. For histochemical staining of β-galactosidase-producing cells, medium was removed 48 h p.i., and then cells were washed twice with PBS and briefly fixed in 0·2% glutaraldehyde–2% formaldehyde. After washing, cells were incubated in a staining solution containing 0·6 mg/ml chromogenic substrate 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-Gal, Boehringer Mannheim), 5 mM ferrocyanide and 5 mM ferricyanide and 2 mM $MgCl_2$ in PBS at 37 °C for 2 h. Microscopic analysis monitoring for wells containing blue stained cells allowed the determination of virus titres as $TCID_{50}$/ml.

Previous work demonstrated that a variety of mammalian cells lines (of human, bovine, equine, canine or rodent origin) are non-permissive for MVA replication. Only the African green monkey kidney cell line MA104 supported MVA growth under low-multiplicity growth conditions (Meyer *et al.*, 1991). In one-step growth experiments performed for this

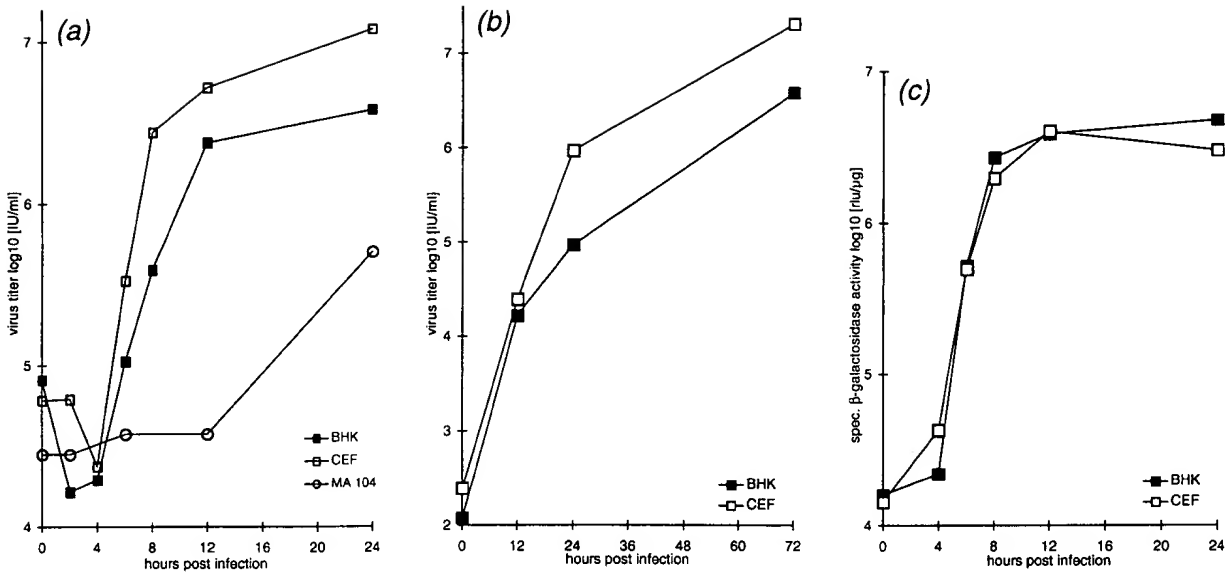


Fig. 1. MVA growth and recombinant gene expression in BHK cells. One-step growth curves were established after infection of BHK, MA104 or CEF cells with 10 IU per cell MVA-LZ (*a*). Low-multiplicity growth of MVA was analysed after infection of BHK cells or CEF with 0·05 IU per cell MVA-LZ (*b*). Cell cultures were harvested at the indicated time-points after infection. Virus titres were determined by titration on CEF. Synthesis of recombinant β-galactosidase in BHK cells and CEF was compared (*c*). Cells were infected with 10 IU per cell MVA-LZ and harvested at multiple time-points after infection. Cytoplasmic extracts were prepared and specific β-galactosidase activities (relative light units/µg total protein) were determined.

study, MVA could also be propagated in the kidney cell lines CV-1 and BHK from monkey and hamster, respectively. While CV-1 and MA104 cells produced at best about one-tenth the amount of virus compared to CEF cells, BHK were far more permissive (Table 1). That BHK cells produced significant virus titres was worth further investigation. Very similar virus replication profiles were observed with multiple time-point one-step growth kinetics in BHK and CEF cells (Fig. 1*a*). Even the timing of the post-adsorption lag phase is identical in both cell lines, whereas productive infection of MVA in MA104 cells leads to rather delayed and reduced increase of virus titres. Experiments using low multiplicities of infection also revealed similar kinetics of MVA replication in BHK and CEF cells, particularly within the first 12 h (Fig. 1*b*). These data suggest that assembly of mature virions is not significantly different or delayed in BHK cells compared to CEF cells. Interestingly, low-dose MVA infection of BHK cell monolayers resulted in formation of typical foci of infected cells which remained attached to the tissue culture plates (data not shown). Expression of the recombinant gene *lacZ* under the control of the vaccinia virus late promoter P11 was also monitored, including detailed kinetics of β-galactosidase production in BHK and CEF cells infected with 10 IU per cell of MVA-LZ. After incubation of infected cultures for various times (0, 4, 6, 8, 12 and 24 h), the specific enzyme activity in the prepared cytoplasmic extracts was determined (Miller, 1972) and proved to be almost identical in both BHK and CEF (Fig. 1*c*). These data support the use of BHK cell monolayers for isolation of recombinant MVA expressing the *lacZ* marker gene.

Of particular interest for *in vivo* applications of live MVA vaccines in humans is its replication profile and recombinant gene expression in potential target cells. Although shown to be replication-deficient in multiple transformed cell lines of human origin (Table 1) investigation of MVA virus production in primary human cells would provide another stringent test for its clinical safety. Primary cells with particular capacities in antigen-presentation such as DC or blood monocytes could represent important target cells for clinical trials using *ex vivo* infection of patient cells for immune therapy. After *in vitro* infection, these cells would produce and present the desired antigens and either be retransferred to the patient to stimulate antigen-specific immune responses or used for *in vitro* induction or amplification of T-cells which can than be adoptively transferred (Boon *et al.*, 1995; Rosenberg, 1996; Girolomoni & Ricciardi-Castagnoli, 1997). The approval of such clinical protocols might be significantly accelerated if any productive replication of the vector virus in target cells can be excluded. Blood-derived cells or primary skin fibroblasts representing ideal target cells for such clinical trials were used in our experiments. Although abortive infection of primary human macrophages or DC has been demonstrated even with replication-competent vaccinia virus strains (Broder *et al.*, 1994; Bronte *et al.*, 1997), PBMC and HF are reasonably permissive for vaccinia virus multiplication. In contrast, by analysing virus multiplication under one-step growth conditions we could demonstrate the inability of MVA to replicate in all primary human cells tested (Fig. 2*a*). In addition to the detailed kinetics of virus growth, histochemical β-galacto-

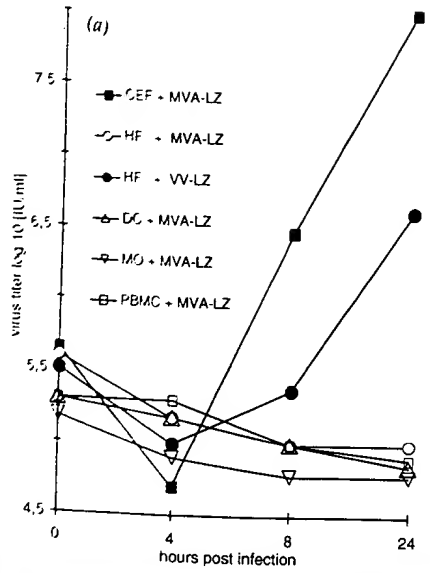
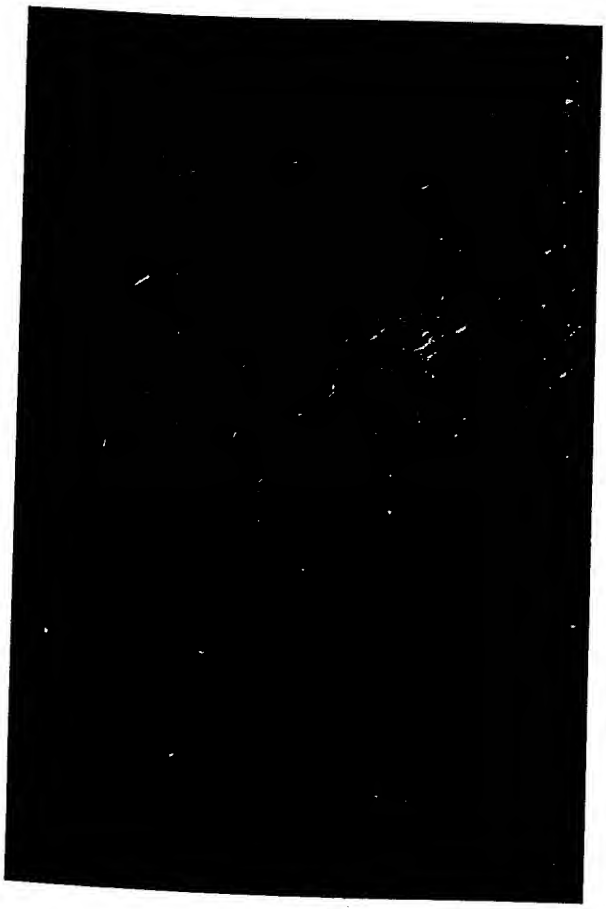
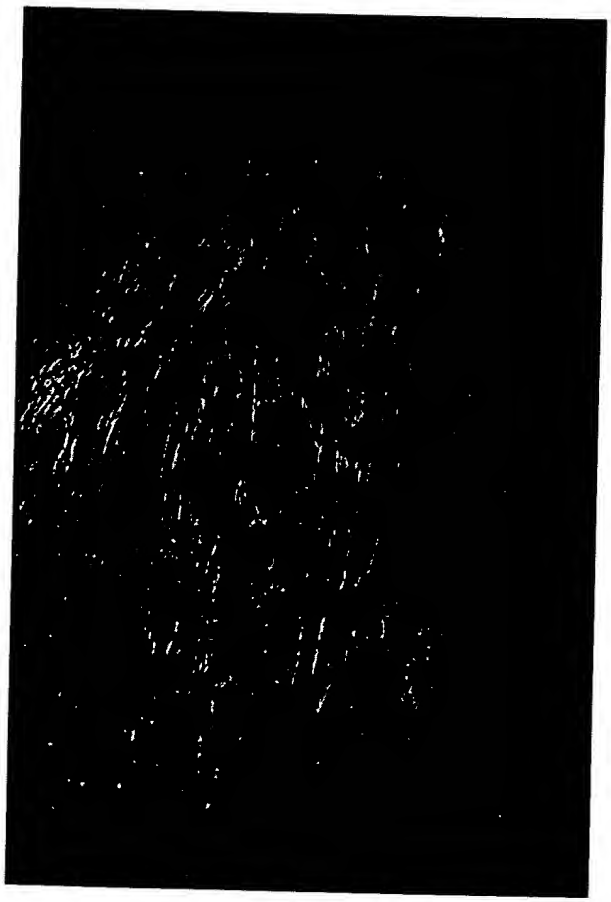


Fig. 2. MVA multiplication in primary human cells. One-step virus growth curves are shown for CEF cells and for primary human cells (HF, DC, MO and PBMC) (*a*). Virus growth was analysed after infection of cells with 10 IU per cell MVA-LZ or VV-LZ. Virus titres in cells harvested at 0, 4, 8 and 24 h after infection were determined by back-titration on CEF. (*b*, *c*) Micrographs of HF monolayers infected with a low m.o.i. of MVA-LZ (*b*) and VV-LZ (*c*). At 48 h p.i., the cells were fixed and stained histochemically with X-Gal. Three single cells infected with MVA-LZ are marked by arrows. One single virus plaque formed by VV-LZ is shown.

(*b*)



(*c*)



sidase-specific staining of HF monolayers infected at low-multiplicity confirmed that MVA infection was non-permissive for productive replication but allowed efficient expression of recombinant genes regulated by the vaccinia virus late promoter P11 (Fig. 2*b*). In contrast, VV-LZ replicated well in HF forming large lytic plaques in the cell monolayer (Fig. 2*c*).

In summary, the screening for virus production in various human and animal cells recommends BHK cells as an accessible alternative to primary CEF cells for routine MVA virus propagation and manipulation. BHK cell lines are routinely used in many laboratories, and even licensed in some cases for vaccine production. Although high virus titres were obtained from the BHK cell line, there was no evidence of significant MVA virus production in various other human or animal cell lines chosen for this investigation. Another important finding was the complete inability of MVA to productively replicate in all primary human cells tested. Together with the existing evidence that MVA is a very safe live vaccine in humans, these results make the handling and testing of recombinant MVA virus much more accessible to a variety of experimental and clinical research projects aimed at developing prophylactic vaccination and therapeutic treatment of infectious diseases and cancer.

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References

Baron, M. D. & Barrett, T. (1997). Rescue of rinderpest virus from cloned cDNA. *Journal of Virology* **71**, 1265–1271.

Boon, T., Gajewski, T. F. & Coulie, P. (1995). From defined human tumor antigens to effective immunization? *Immunology Today* **16**, 334–336.

Brack-Werner, R., Kleinschmidt, A., Ludvigsen, A., Mellert, W., Neumann, M., Herrmann, R., Khim, M. C. L., Burny, A., Müller-Lantzsch, N., Stavrou, D. & Erfle, V. (1992). Infection of human brain cells by HIV-1: restricted virus production in chronically infected human glial cell lines. *AIDS* **6**, 273–285.

Broder, C. C., Kennedy, P. E., Michaels, F. & Berger, E. A. (1994). Expression of foreign genes in cultured human primary macrophages using recombinant vaccinia virus vectors. *Gene* **142**, 167–174.

Bronte, V., Carroll, M. W., Goletz, T. J., Wang, M., Overwijk, W. W., Marincola, F., Rosenberg, S. A., Moss, B. & Restifo, N. P. (1997). Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. *Proceedings of the National Academy of Sciences, USA* **94**, 3183–3188.

Carroll, M. W., Overwijk, W. W., Chamberlain, R. S., Rosenberg, S. A., Moss, B. & Restifo, N. P. (1997). Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: a murine tumor model. *Vaccine* **15**, 387–394.

Collins, P., Hill, M., Camargo, E., Grosfeld, H., Chanock, R. & Murphy, B. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proceedings of the National Academy of Sciences, USA* **92**, 11563–11567.

Girolomoni, G. & Ricciardi-Castagnoli, P. (1997). Dendritic cells hold promise for immunotherapy. *Immunology Today* **18**, 102–104.

Fenner, F, Henderson, D. A., Arita, I., Jezek, Z. & Ladnyi, I. D. (1988). *Smallpox and its Eradication*, pp. 539–592. Geneva: World Health Organization.

Hirsch, V., Fuerst, T., Sutter, G., Carroll, M., Yang, L., Goldstein, S., Piatak, M., Elkins, W., Alvord, G., Montefiori, D., Moss, B. & Lifson, J. (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *Journal of Virology* **70**, 3741–3752.

Mackett, M., Smith, G. & Moss, B. (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proceedings of the National Academy of Sciences, USA* **79**, 7415–7419.

Mahnel, H. & Mayr, A. (1994). Erfahrungen bei der Schutzimpfung gegen Orthopocken von Mensch und Tier mit dem Impfstamm MVA. *Berliner und Münchener Tierärztliche Wochenschrift* **107**, 253–256.

Mayr, A., Hochstein-Mintzel, V. & Stickl, H. (1975). Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. *Infection* **3**, 6–14.

Mayr, A., Stickl, H., Müller, H., Danner, K. & Singer, H. (1978). Der Pockenimpfstamm MVA: Marker, genetische Struktur, Erfahrungen mit der parenteralen Schutzimpfung und Verhalten im abwehrgeschwächten Organismus. *Zentralblatt für Bakteriologie und Hygiene, I. Abt. Orig. B* **167**, 375–390.

Mellert, W., Kleinschmidt, A., Schmidt, J., Festl, H., Emler, S., Roth, W. K. & Erfle, V. (1990). Infection of human fibroblasts and osteoblast-like cells with HIV-1. *AIDS* **4**, 527–535.

Meyer, H., Sutter, G. & Mayr, A. (1991). Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *Journal of General Virology* **72**, 1031–1038.

Miller, J. H. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moss, B. (1996). Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proceedings of the National Academy of Sciences, USA* **93**, 11341–11348.

Moss, B., Carroll, M., Wyatt, L., Bennink, J., Hirsch, V., Goldstein, S., Elkins, W., Fuerst, T., Lifson, J., Piatak, M., Restifo, N., Overwijk, W., Chamberlain, R., Rosenberg, S. & Sutter, G. (1996). Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. In *Novel Strategies in the Design and Production of Vaccines*, pp. 7–13. Edited by S. Cohen & A. Shafferman. New York: Plenum Press.

Panicali, D. & Paoletti, E. (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proceedings of the National Academy of Sciences, USA* **79**, 4927–4931.

Rosenberg, S. A. (1996). Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens. *Journal of the National Cancer Institute* **88**, 1635–1644.

Schneider, H., Spielhofer, P., Kaelin, K., Dötsch, C., Radecke, F., Sutter, G. & Billeter, M. A. (1997). Rescue of measles virus using a replication-deficient vaccinia-T7 vector. *Journal of Virological Methods* **64**, 57–64.

Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H., Schäfer, H. & Holzner, A. (1974). MVA-Stufenimpfung gegen Pocken. *Deutsche medizinische Wochenschrift* **99**, 2386–2392.

Sutter, G. & Moss, B. (1992). Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proceedings of the National Academy of Sciences, USA* **89**, 10847–10851.

Sutter, G. & Moss, B. (1995). Novel vaccinia vector derived from the host range restricted and highly attenuated MVA strain of vaccinia virus. *Developments in Biological Standardization* **84**, 195–200.

Sutter, G., Wyatt, L. S., Foley, P. L., Bennink, J. R. & Moss, B. (1994). A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* **12**, 1032–1040.

Sutter, G., Ohlmann, M. & Erfle, V. (1995). Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Letters* **371**, 9–12.

Werner, G. T., Jentzsch, U., Metzger, E. & Simon, J. (1980). Studies on poxvirus infections in irradiated animals. *Archives of Virology* **64**, 247–256.

Wyatt, L., Moss, B. & Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**, 202–205.

Wyatt, L. S., Shors, S. T., Murphy, B. R. & Moss, B. (1996). Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* **14**, 1451–1458.

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Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model

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The highly attenuated, replication-deficient, modified vaccinia virus Ankara (MVA) was used to express the fusion (F) and/or hemagglutinin-neuraminidase (HN) glycoproteins of parainfluenza virus 3 (PIV3). Initial recombinant viruses in which the HN gene was regulated by a very strong synthetic early/late promoter replicated poorly in permissive chick embryo cells evidently due to toxic levels of the gene product. This result led us to construct and evaluate a modified early/late promoter derived from the H5 gene of vaccinia virus. Reporter gene experiments indicated that the enhanced H5 promoter was about five times stronger than the 7.5 promoter used in previous recombinant vaccinial PIV3 viruses. Although the overall expression from the modified H5 promoter was less than that of the strong synthetic promoter, early expression, determined in the presence of an inhibitor of DNA replication, was higher. Importantly, recombinant MVA employing the modified H5 promoter to regulate the F or HN gene of PIV3 replicated to high titers in chick cells and expressed functional F or HN proteins as measured by syncytial formation upon dual infection of mammalian cells. Cotton rats inoculated with recombinant MVA expressing F or HN by intramuscular or intranasal routes produced high levels of antibody. The virus expressing HN, however, was the more effective of the two in inducing immunity to PIV3 challenge, reducing PIV3 viral titers in the nasal turbinates by at least 4.7 logs and in the lungs by 3.4 logs, similar to that achieved by immunization with PIV3. These studies support further testing of recombinant MVA/PIV3 viruses as safe and effective candidate vaccines. Copyright © 1996 Elsevier Science Ltd.

Keywords: Parainfluenza virus; vaccinia virus; MVA strain

A safe and effective vaccine for disease caused by parainfluenza viruses (PIV), the second most important viral pathogen of the lower respiratory tract in young children, has long been sought. Inactivated parainfluenza virus 3 (PIV3) vaccines have been unsuccessful[1], but live attenuated PIV3 vaccine candidates, both bovine and human, are currently under study[2]. Less traditional PIV vaccines such as recombinant vaccinia viruses (VV) have also been considered. Intradermal (i.d.) inoculations of VV, strain WR, expressing the fusion (F) and hemagglutinin-neuraminidase (HN) genes of PIV3 induced protective immunity in cotton rats[3] and patas monkeys[4]. Nevertheless, safety concerns, including the potential ability of VV to spread in an immunocompromised individual, discouraged further testing of the vaccine.

There was concern with the safety of VV towards the end of the smallpox era, and several highly attenuated vaccines were developed[5]. One of these, modified VV Ankara (MVA), was derived by over 500 passages in chick embryo cells resulting in multiple genomic deletions and an inability to replicate in mammalian cells[6,7]. MVA is nonpathogenic in experimental animals and was administered without untoward incidence to 120000 humans, many of whom were considered poor risks for the standard vaccine[6,8]. The greatly impaired ability to replicate in mammalian cells was shown to result from a block in virion morphogenesis and importantly had no apparent effect on viral or recombinant gene expression[9,10]. Indeed, immunization of mice with a recombinant MVA that expressed the influenza virus hemagglutinin and nucleoprotein genes protected them against lethal doses of influenza virus as well as did

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recombinants constructed from a standard New York City Board of Health vaccine strain VV[10].

We now describe the construction and characterization of recombinant MVA viruses that express PIV3 F and HN genes and their successful use for intramuscular (i.m.) or intranasal (i.n.) immunization of cotton rats against a respiratory PIV3 challenge.

MATERIALS AND METHODS

Viruses and cells

Stocks of VV strain WR were propagated in BS-C-1 and HeLa cells as described[11]. MVA and MVA recombinant stocks were prepared in secondary chicken embryo fibroblasts (CEF) in minimal essential medium supplemented with 2% fetal bovine serum[10]. PIV3 strain 47885 was propagated in HEp-2 cells.

Plasmid constructions

The F and HN genes of PIV3 were excised from the plasmids pTZ18R and pGEM3Zf+ by BamHI digestion[3]. The ends of the F gene were blunted by incubation with Klenow enzyme and deoxynucleoside triphosphates and cloned into the SnaBI site in pJS-5 which contains two strong synthetic VV early/late promoters (Chakrabarti *et al.*, manuscript in preparation). A plasmid insertion vector with both F and HN genes was made by inserting the BamHI digested HN gene into the BamHI site in pJS-5 (pLW-1). The double promoter cassette containing the F and HN genes was transferred to G06, a plasmid containing MVA flanks for insertion into del III of MVA[10], and the resulting plasmid was designated pLW-2.

A plasmid insertion vector with a single synthetic promoter (pLW-7) was made by SmaI and HindIII digestion of GO6, blunting with Klenow enzyme, and inserting this single synthetic promoter fragment into the SmaI site of G02. This GO2 plasmid is similar to pIII, with MVA flanks for insertion into del III, but also contains the *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene outside the flanks of the MVA DNA under the control of the 7.5 promoter[9]. The blunt-ended BamHI digested F and HN genes were put into the PmeI site of pLW-7 to make a PIV F construct (pLW-10) and a PIV HN construct (pLW-11) each controlled by a single synthetic promoter.

A modified H5 promoter (from the H5 gene of VV WR) was engineered by annealing overlapping oligonucleotides. Two alterations in base-pair sequence from the natural promoter were purposely made as shown in *Figure 1*B. T was substituted for A in position −102 and A was substituted for T at position −38 for reasons indicated later. The modified H5 promoter was cloned into vector G02 (described above), and the resulting plasmid was designated pLW-9. The BamHI digested F and HN genes were cloned singly into pLW-9 to produce pLW-12 and pLW-13, respectively.

We made two plasmids in which the *E. coli lac Z* gene is controlled by the modified H5 promoter (pLW-19) or the 7.5 promoter (pLW-20). For pLW-19, pLW-9 was digested with PstI and HincII, and the H5 promoter fragment was blunted with Klenow enzyme, and ligated into pSC65 (Chakrabarti *et al.*, manuscript in preparation) from which the strong synthetic and 7.5 promoters had been removed by SalI and XhoI digestion. For pLW-20, the strong synthetic promoter was removed from pSC65 by excision with HindIII and SmaI, and the plasmid was blunted, and religated, leaving the $P_{7.5}$ controlling the *lac Z* gene.

Generation of recombinant viruses

Recombinant VV, strain WR, or MVA viruses were made by infection of BS-C-1 or CEF six well (35 mm) plates, respectively, with a multiplicity of infection of 0.05 p.f.u. cell^{-1}. The infected cells were transfected 90 min later with 10 μg of plasmid DNA per well using Lipofectin (Gibco, BRL) as recommended by the manufacturer. After 48 h of infection, the cells were harvested as described[11]. Recombinant VV strain WR which expressed the PIV F and HN (vLW-1) was obtained by repeated picking of syncytial plaques in BS-C-1 cells. Recombinant VV strain WR viruses which expressed β-galactosidase controlled by either the H5 or 7.5 promoter (vLW-19 and vLW-20, respectively) were obtained by repeated picking of blue-staining β-galactosidase-producing plaques[11]. MVA recombinants (vLW-2, vLW-10, vLW-11, vLW-12, and vLW-13) which expressed PIV F and/or HN were detected by immunostaining of virus plaques (foci of morphologically altered cells, instead of clear plaques as with the WR strain) utilizing polyclonal anti-PIV3 rabbit serum (Accurate Scientific) followed by anti-rabbit Ig conjugated to peroxidase (Amersham, Arlington Heights, IL) and *O*-dianisidine (Sigma, St Louis, MO) as substrate. vLW-12 and vLW-13 were subsequently renamed MVA/PIV_F and MVA/PIV_{HN} and used in the animal experiments.

Radioimmunoprecipitation of virus-infected cell lysates

Secondary CEF cells in 25 cm^2 flasks were infected with a multiplicity of 10 infectious units ml^{-1} of recombinant VV. At 2 h post-infection, the virus inoculum was replaced with methionine-free minimal essential medium containing 5% dialyzed fetal calf serum and 50 μCi of [^{35}S]methionine per ml and incubated for 14 h. Cells were lysed in buffer containing 0.15 M NaCl, 0.01 M Tris HCl (pH 7.4), 1% sodium deoxycholate, 1% Triton X-100, and incubated overnight with anti-PIV polyclonal rabbit serum (Accurate Scientific), followed by 20% protein A-Sepharose suspension. Immune complexes were washed in the above buffer, proteins from the reaction were resuspended in Laemmli buffer, and resolved by electrophoresis in a 10% SDS-polyacrylamide gel.

Animal immunization and challenge

Groups of six young adult cotton rats (*Sigmodon hispidus*) were immunized i.m. or i.n. with 10^8 infectious units of wild type MVA or MVA recombinant, or i.n. with 10^6 PFU of PIV3 in a 0.1 ml amount. At 28 days after immunization, the animals were bled and then boosted with the same dose of MVA or MVA recombinant. PIV immunized animals were not boosted. The animals were bled 50 days after the initial immunization and next challenged with 10^5 p.f.u. of PIV3 i.n. on day

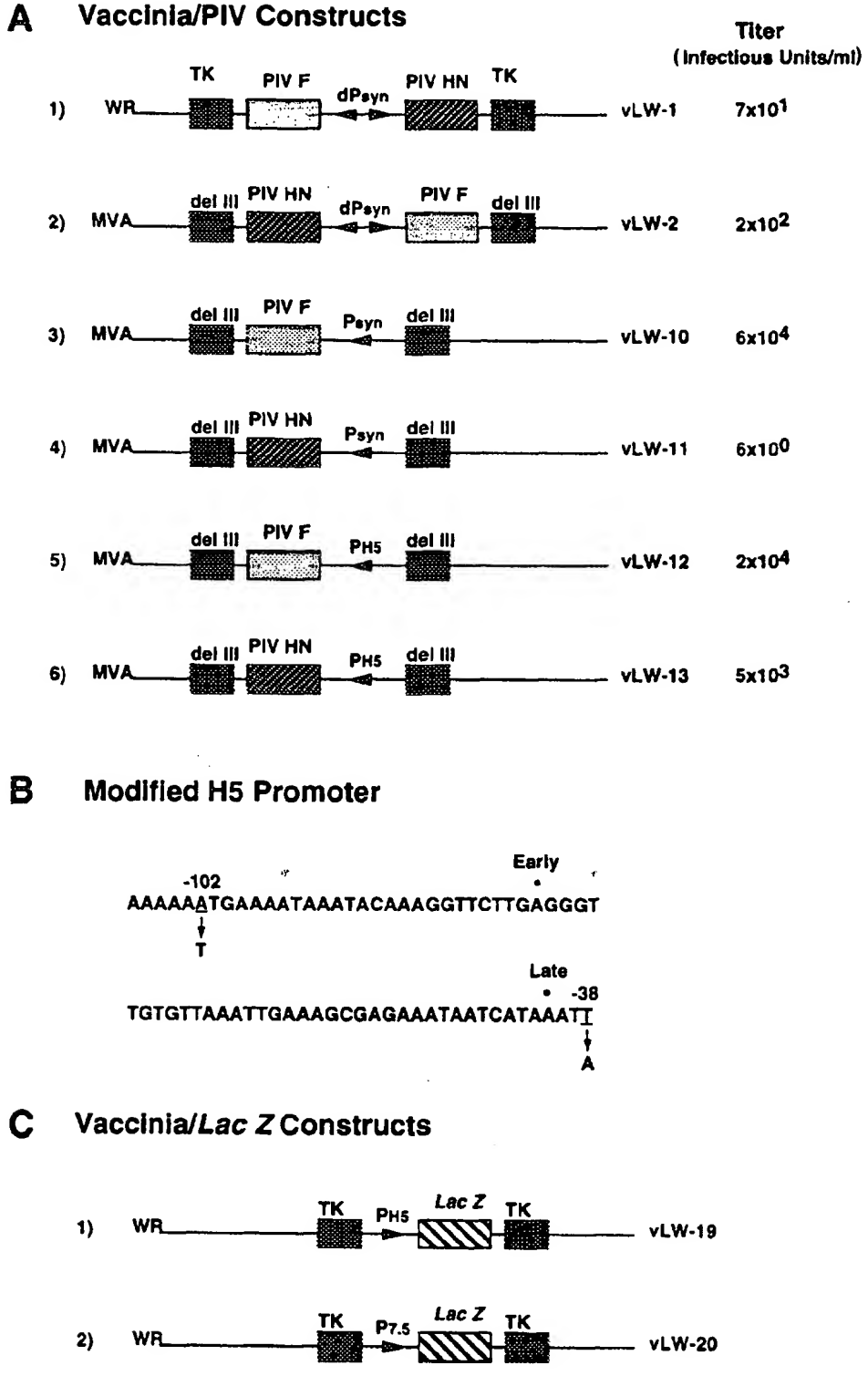

Figure 1 Recombinant VV. (A) Representation of WR and MVA recombinant VV genomes containing PIV3 F and/or HN genes. Promoter abbreviations: P_{syn}, single synthetic promoter; dP_{syn}, double synthetic promoters; P_{H5}, modified H5 promoter. Arrowheads indicate the direction of transcription. Flanking sequences at insertion site: TK, thymidine kinase; del III, a 3500 bp deletion in the HindIII A fragment of the MVA genome. Virus titers as given are from an average of at least three individual plaques that had been plaque-purified four or five times in succession. Virus suspensions were made from individual plaques at 48 h after infection. Titers of suspensions were determined by immunostaining at 48 h post-infection. vLW-12 and vLW-13 were renamed MVA/PIV_F and MVA/PIV_{HN} and used in animal experiments. (B) Sequence of the VV (strain WR) H5 promoter; arrows point to nucleotide substitutions made. Nucleotides are numbered from the A in the ATG start site of the H5 gene. The dots are over the early and late RNA start sites determined by Rosel *et al.*[14] (C) Representation of genomes of recombinant VV expressing *lacZ* reporter gene under control of a natural 7.5 or modified H5 promoter

63. Four days after challenge, the nasal turbinates and lungs of each cotton rat were removed and virus titer ($TCID_{50}$/g) was determined[3].

Immunological assays

Hemagglutination-inhibition (HI) and ELISA assays to measure PIV specific antibody responses were performed as previously described[12,13].

RESULTS

Construction of recombinant MVA expressing the PIV3 HN and F genes

Stable recombinant MVA expressing both the influenza virus HA and NP genes regulated by the strong synthetic early/late promoter (P_{syn}) and inserted into deletion region III had previously been made[10]. The analogous recombinant MVA expressing the PIV3 F and HN genes (vLW-2), (*Figure 1*A, 2) could only be amplified to relatively low titer in CEF cells (10^8 infectious units ml^{-1}). Titers of the WR recombinant expressing the two PIV3 genes regulated by P_{syn} inserted into the TK region (vLW-1) (*Figure 1*A, 1) in BS-C-1 cells were even lower than those of the above MVA/PIV recombinant virus. Since related results were obtained with two different strains of VV, different insertion sites, and different host cells, we suspected that high expression of one or both of the PIV3 genes inhibited VV replication. We therefore made a new MVA transfer vector, pLW-7, that contains a single P_{syn} and used it to make recombinant MVA expressing either the PIV3 F (vLW-10) or HN (vLW-11) genes (*Figure 1*A, 3,4). Recombinant MVA expressing PIV3 F grew well but the yield of MVA expressing PIV3 HN was very low. We noted that the amount of infectious virus recovered within a single plaque or focus was predictive of the ability to subsequently prepare high titer virus stocks (1×10^9 infectious units ml^{-1}). The average titer of virus suspension from a single plaque after four or five successive plaque isolations is given (*Figure 1*A, 1–6).

Since a recombinant WR strain VV expressing the PIV3 HN under the relatively weak 7.5 promoter had previously been constructed[3], we considered that the present problem was related to use of the very strong P_{syn}. We decided to test other promoters in order to derive one that provided a level of expression intermediate between $P_{7.5}$ and P_{syn}. Rosel *et al.*[14] had described the early/late promoter preceding the H5 (previously called H6) gene, and a version of this has been used by others[15–17] for recombinant VV gene expression. Based on previous studies[18], we made an A→T substitution to alter an upstream ATG codon without adversely affecting expression and a T→A substitution to place a preferred purine downstream of the transcription initiation site (*Figure 1*B). This modified promoter will be referred to as P_{H5}.

In order to compare the activity of the modified H5 promoter with other promoters, we constructed a WR strain recombinant VV with *lacZ* as a reporter gene controlled by P_{H5}, vLW-19 (*Figure 1*C, 1). A similar recombinant VV with the reporter gene controlled by $P_{7.5}$, vLW-20, was also made. In each case, two independent clones were plaque purified and amplified. We also tested previously made vSC8 and vSC56 viruses in which the *lacZ* gene is controlled by a strong late (P_{11}) promoter and the P_{syn}, respectively. Cells were infected in the presence of Ara-C, an inhibitor of DNA replication, to measure early promoter activity or in the absence of drug to measure combined early/late or late promoter activities. Representative data from such experiments are shown in *Table 1*. In the absence of drugs, the activities of the promoters were $P_{syn}P_{11}P_{H5}P_{7.5}$. In the presence of Ara-C, the order was $P_{H5}P_{syn}P_{7.5}P_{11}$. Thus, P_{H5} is a strong early and a moderate late promoter.

Table 1 Strength of VV promoters as measured by *E. coli lacZ* expression

Virus	Promoter	β-gal synthesis[a] (μg) +Ara-C	β-gal synthesis[a] (μg) −Ara-C
vLW-20 (1)	$P_{7.5}$ (E/L)	0.21	0.52
vLW-20 (2)	$P_{7.5}$ (E/L)	0.20	0.55
vLW-19 (1)	P_{H5} (E/L)	1.03	3.05
vLW-19 (2)	P_{H5} (E/L)	1.16	2.14
vSC8[b]	P_{11} (L)	0	3.86
vSC56[c]	P_{syn} (E/L)	0.32	8.24

[a]BS-C-1 cells in 24-well plates were infected at a multiplicity of infection of 10 with virus containing the indicated natural, modified, or synthetic VV early/late (E/L) or late (L) promoter regulating β-galactosidase expression in the presence or absence of 44 mg ml^{-1} of Ara-C. In the case of vLW-20 and vLW-19, two independently isolated recombinant viruses designated (1) and (2) were tested. The cells were lysed at 24 h and total β-galactosidase activity was measured spectrophotometrically[32]. [b]vSC8[33]. [c]vSC56 (Chakrabarti *et al.*, manuscript in preparation)

We decided to use the modified H5 promoter to make recombinant MVA expressing the PIV3 genes, since the level of expression should be less than that achieved with P_{syn}. A new transfer vector pLW-9 containing a single P_{H5} was used to clone the F and HN genes and two separate recombinant viruses, vLW-12 (MVA/PIV_F) and vLW-13 (MVA/PIV_{HN}) were isolated (*Figure 1*A, 5 and 6). These viruses replicated well and high titer stocks were prepared (10^9 infectious units ml^{-1}).

Synthesis and biological activity of recombinant proteins in mammalian cells

CEF were infected with MVA/PIV_F or MVA/PIV_{HN} and metabolically labeled with [^{35}S]methionine. Infected cell lysates were prepared, and the PIV proteins were immunoprecipitated with polyclonal PIV3 antiserum and then analyzed by SDS PAGE. Two major bands of about 60 and 51 kDa corresponding to the F_0 precursor and F_1 glycoproteins were detected by autoradiography of MVA/PIV_F immunoprecipitates (*Figure 2*). A 9000 kDa F_2 subunit was not resolved on this gel. A major HN polypeptide of about 70000 kDa was visualized (*Figure 2*). The electrophoretic mobilities of the PIV3 proteins made by MVA recombinants were similar to those made by previous VV WR recombinants. MVA expressed larger amounts of recombinant protein than WR presumably due to the greater strength of the H5 promoter compared to the 7.5 promoter. Similar F and HN protein bands were observed in mammalian BS-C-1 and HeLa cells (data not shown).

Synthesis of both the PIV 3 F and HN proteins is needed for syncytium formation[19–21]. Thus, no syncytia were formed upon infection of BS-C-1 cells with either MVA/PIV_F or MVA/PIV_{HN} (*Figure 3*B and C). The

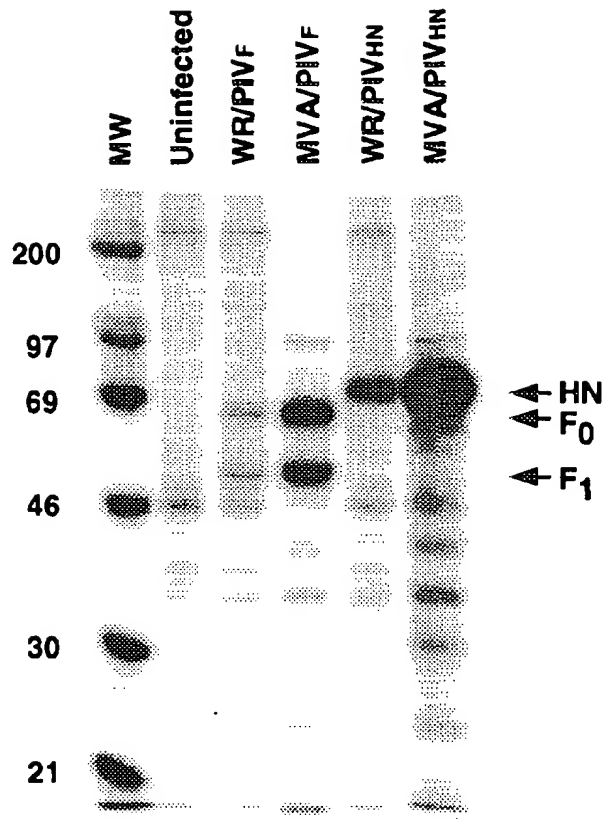

Figure 2 Expression of recombinant proteins. Infected cells were metabolically labeled with [^{35}S]methionine and lysates were immunoprecipitated with PIV3 antiserum. Arrows denote the F_0 precursor and the F_1 subunit of the PIV F protein and the HN protein of PIV3. Lane marked MW contains standard protein markers with molecular weights $\times 10^{-3}$ indicated on the side

formation of syncytia upon dual infection provided evidence for the biological activities of both proteins (*Figure 3*D).

Antibody responses of cotton rats to MVA/PIV recombinants

Cotton rats were inoculated with 10^8 infectious units of each recombinant virus, boosted at 1 month, and the level of serum antibodies induced by the individual glycoproteins was assessed. The animals exhibited no untoward effects due to the vaccine, and HI titers at 28 days indicated good antibody responses to the MVA/PIV$_{HN}$ inoculated via i.m. or i.n. routes (*Table 2*). The high ELISA titers obtained with purified PIV3 as the antigen confirmed these results and also showed that MVA/PIV$_F$ was immunogenic by both routes (*Table 3*). In addition, in each case except the i.n. inoculation of MVA/PIV$_F$, the ELISA titer was higher after the boost.

Protection against PIV3 infection

Cotton rats were challenged with PIV3 at 63 days after initial immunization. After 4 days, PIV3 titers of nasal turbinates and lungs from killed animals were determined (*Table 4*). Protection afforded by MVA/PIV$_F$ was observed in both i.m. and i.n. inoculated cotton rats; the PIV3 titers of nasal turbinates and lungs were 100- to 1200-fold lower than with the MVA controls. MVA/PIV$_{HN}$ offered even greater protection (2500–63000 fold reduction in PIV3 titers) similar to that conferred by prior inoculation with PIV3. i.m. and i.n. inoculations were equally effective in inducing resistance to replication of challenge virus.

DISCUSSION

A previous report[3] described the ability of recombinant VV expressing F and HN glycoproteins of PIV3 to induce resistance to replication of PIV3 in cotton rats. However, VV strain WR is not acceptable as a vector because of its passage history and even standard VV vaccine strains have potential to spread in immunocompromised hosts[5,22]. For such reasons, we and others have explored the use of attenuated VV[23–25] or other poxviruses[26] as vectors. We have focused on the MVA strain because of its inability to replicate efficiently in mammalian cells and safety record for human vaccination[7–9,27]. Remarkably, MVA retains the ability to express genes in mammalian cells at the same level as replication competent VV strains[9,10], a feature not directly demonstrated for other host restricted poxviruses.

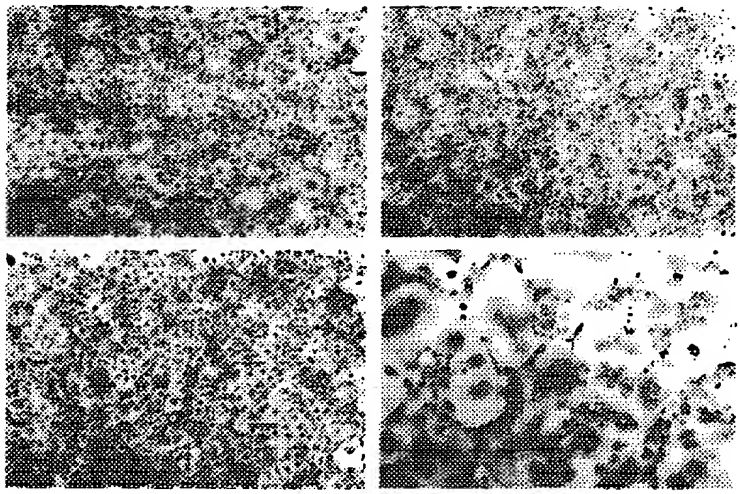

Figure 3 Cell fusion following co-infection of BS-C-1 cells with recombinant MVA expressing F and HN proteins. Cells were infected at an MOI of 1 with (A) MVA (B) MVA/PIV$_F$, (C) MVA/PIV$_{HN}$, or (D) MVA/PIV$_F$ and MVA/PIV$_{HN}$ together. At 24 h, the cells were fixed, stained with crystal violet, and photographed

Table 2 HI serum antibody response to immunization with MVA/PIV3 recombinant or wild type PIV3

Immunizing virus	HI titer[a] on indicated day:[b] i.m.[c] 0	28	50	i.n.[c] 0	28	50
MVA	<2	<2	<2	<2	<2	<2
MVA/PIV$_F$	<2	<2	<2	<2	<2	<2
MVA/PIV$_{HN}$	<2	9.2	9.8	<2	7.3	8.8
MVA/PIV$_F$+MVA/PIV$_{HN}$	<2	8.4	9.0	<2	7.2	8.0
PIV3	—	—	—	<2	8.5	9.7

[a]Reciprocal serum mean $\log_2$ antibody titer (N=6 per group). [b]Cotton rats were boosted on day 28. PIV3 group was not boosted. [c]i.m.=intramuscular route of virus administration, i.n.=intranasal

Table 3 ELISA serum antibody response of cotton rats to immunization with MVA/PIV3 recombinant virus or wild type PIV3

Immunizing virus	ELISA titer[a] on indicated day:[b] i.m.[c] 0	28	50	i.n.[c] 0	28	50
MVA	7.6	8.6	8.6	9.3	9.0	8.6
MVA/PIV$_F$	7.3	11.8	13.3	8.9	13.7	13.7
MVA/PIV$_{HN}$	7.6	13.3	14.3	8.8	12.3	14.3
MVA/PIV$_F$+MVA/PIV$_{HN}$	8.9	12.5	14.5	7.6	13.3	15.3
PIV3	—	—	—	8.3	15.6	13.3

[a]Reciprocal serum mean $\log_2$ antibody titer (N=6 per group); purified PIV3 virions were used as the solid phase antigen. [b]Cotton rats were boosted on day 28. PIV3 group was not boosted. [c]i.m.= intramuscular route of virus administration, i.n.=intranasal

In the present study, recombinant MVA expressing the F and HN glycoproteins of PIV3 were constructed. Unexpectedly, we were unable to prepare high titer stocks of recombinant MVA expressing the HN alone or together with the F regulated by strong synthetic promoters even though similar constructs expressing influenza HA and NP and PIV3 F could be produced. We had similar problems in trying to make the analogous WR strain vaccinia virus using a different site of integration. In this case, however, the use of a fusogenic cell line (BS-C-1) may have contributed to the problem. Since a recombinant VV expressing HN with the relatively weak 7.5 promoter had been easily constructed[3], we considered that overexpression of HN interferes with VV replication. Since MVA cannot spread, it seemed advantageous to use a stronger promoter than $P_{7.5}$. We evaluated a promoter derived from the H5 (previously called H6) gene[14] because it has early and late elements, was well characterized[14], and was used for recombinant gene expression[15-17]. Based on the extensive promoter mutagenesis studies of Davison and Moss[18] we made two nucleotide substitutions to remove a potential translation initiation codon and to enhance promoter strength. As anticipated, the overall expression obtained with the modified H5 promoter was intermediate between the $P_{7.5}$ and the P_{syn}. However, the early promoter element of P_{H5} was three to fivefold stronger than either of the others. Because of the reported importance of early expression in the initiation of the cellular immune response[28,29], the modified H5 promoter may prove advantageous for constructing vaccine candidates. We therefore made transfer vectors containing the modified H5 promoter which should be of general use. Importantly, recombinant MVA expressing PIV HN or F under control of P_{H5} were stable and replicated to high titers in chick embryo cells. Moreover, these MVA recombinants produced more HN and F than previous replication-competent WR strain viruses expressing HN or F. The biological activity of each PIV3 protein was demonstrated by fusion of mammalian cells upon co-infection with MVA/PIV$_F$ and MVA/PIV3$_{HN}$.

Cotton rats were selected for study since PIV3 is able to replicate to moderately high titers in their lungs and nasal turbinates[3]. MVA recombinants inoculated i.m. or i.n. elicited good antibody responses which could be boosted upon a second inoculation. Each recombinant protected against PIV3 challenge as measured by a reduced level of replication of PIV3 in the lungs and nasal turbinates. However, MVA/PIV$_{HN}$ was clearly a superior vaccine candidate compared to MVA/PIV$_F$ and induced levels of antibody and resistance that approached that induced by infection with PIV3. The protection obtained with i.m. or i.n. inoculations of MVA/PIV$_{HN}$ was comparable to that previously achieved with i.d. WR/PIV$_{HN}$ but the results with MVA/PIV$_F$ were much better than with WR/PIV$_F$[3]. In the present study, titers of PIV3 in the nasal turbinates of MVA recombinants expressing F showed a 1000-fold reduction whereas only an eightfold reduction was achieved with the WR recombinant in the previous study. Whether the better result was due to a booster immunization or to different VV vectors or promoters cannot be determined at this time.

Table 4 Levels of replication of PIV3 in animals immunized with MVA/PIV$_F$ or MVA/PIV$_{HN}$ recombinant virus[a]

Immunizing virus	Immunization route	Nasal turbinates Viral titer ($\log_{10}$ TCID$_{50}$/g)[b]	Reduction in titer ($\log_{10}$)	Lungs Viral titer ($\log_{10}$TCID$_{50}$/g)	Reduction in titer ($\log_{10}$)
MVA	i.m.	6.2±0.0	—	6.2+0.0	—
MVA/PIV$_F$	i.m.	4.2±0.2	2.0	3.5+0.3	2.7
MVA/PIV$_{HN}$	i.m.	1.4±0.2	4.8	1.9+0.5	4.3
MVA/PIV$_F$+MVA/PIV$_{HN}$	i.m.	1.5±0.5	4.7	2.7+0.5	3.5
MVA	i.n.	6.0±0.1	—	5.4±0.2	—
MVA/PIV$_F$	i.n.	2.9±0.3	3.1	2.7±0.6	2.7
MVA/PIV$_{HN}$	i.n.	1.2±0.2	4.8	2.0±0.1	3.4
MVA/PIV$_F$+MVA/PIV$_{HN}$	i.n.	1.1±0.2	4.9	1.2±0.4	4.2
PIV3	i.n.	1.3±0.4	4.7	≤0.8±0.0	≥4.6

[a]Cotton rats were given 10^8 infectious dose of MVA or MVA recombinant either i.m. or i.n. and boosted with the same dose 1 month later. PIV3 was given once on day 0 at 10^6 p.f.u. The animals were challenged 63 days after original inoculation with 10^5 p.f.u. of PIV3 i.n. Animals were killed four days later. [b]Values are presented±S.E. Minimum level of detectability of virus was 0.8 $\log_{10}$TCID$_{50}$/g

Surprisingly, i.m. inoculations of the VV vectors were as effective as the i.n. route in preventing PIV3 replication even in the nasal turbinates, where one would expect IgA to play an important role. Although we did not measure IgA production, there should be only a low level response following i.m. injection. Therefore, we presume that the protective effect was largely mediated by serum IgG. By contrast, Small *et al.*[30] demonstrated that i.n. inoculation with an appropriate recombinant vaccinia virus prevented replication of influenza virus in both the upper and lower respiratory tract of mice while the i.d. route provided resistance only to influenza virus pneumonia. Similarly, Kanesaki *et al.*[31] reported that enteric immunization with an appropriate recombinant vaccinia virus was not as successful in suppressing viral growth in the upper respiratory tract of cotton rats as i.n. immunization, and attributed resistance at least in part to IgA and cell-mediated cytotoxic activity in the respiratory tract. Importantly, the nonreplicating MVA is able to induce resistance in the upper and lower respiratory tract when administered topically to mucosal nasal membranes.

In summary, i.m. or i.n. administration of a replication-deficient, recombinant MVA vaccine expressing PIV3 HN and to a lesser degree F afforded significant protection against infection with PIV3. The expected safety and the potential efficacy of such a vaccine are encouraging and further studies in non-human primates are warranted.

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REFERENCES

1 Chin, J., Magoffin, R.L., Shearer, L.A., Schieble, J.H. and Lennette, E.H. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. *Am. J. Epidemiol.* 1969, **89**, 449–463

2 Murphy, B.R., Hall, S.L., Kulkarni, A.B. *et al.* An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. *Virus Res.* 1994, **32**, 13–36

3 Spriggs, M.K., Murphy, B.R., Prince, G.A., Olmsted, R.A. and Collins, P.L. Expression of the F and HN glycoproteins of human parainfluenza virus type 3 by recombinant vaccinia viruses: contributions of the individual proteins to host immunity. *J. Virol.* 1987, **61**, 3416–3423

4 Spriggs, M.K., Collins, P.L., Tierney, E., London, W.T. and Murphy, B.R. Immunization with vaccinia virus recombinants that express the surface glycoproteins of human parainfluenza virus type 3 (PIV3) protects patas monkeys against PIV3 infection. *J. Virol.* 1988, **62**, 1293–1296

5 Fenner, F., Henderson, D.A., Arita, I., Jezek, Z. and Ladnyi, I.D. *Smallpox and its Eradication.* World Health Organization, Geneva, 1988, pp. 1–1460

6 Mayr, A., Hochstein-Mintzel, V. and Stickl, H. Abstammung, eigenschaften und verwendung des attenuierten vaccinia-stammes MVA. *Infection* 1975, **3**, 6–14

7 Meyer, H., Sutter, G. and Mayr, A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 1991, **72**, 1031–1038

8 Mayr, A., Stickl, H., Müller, H.K., Danner, K. and Singer, H. Der pockenimpfstamm MVA: Marker, genetische struktur, erfahrungen mit der parenteralen schutzimpfung und verhalten im abwehrgeschwächten organismus. *Zbl. Bakt. Hyg.* 1978, **1671. Abt. Orig. B**, 375–390

9 Sutter, G. and Moss, B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. natn. Acad. Sci. U.S.A.* 1992, **89**, 10847–10851

10 Sutter, G., Wyatt, L.S., Foley, P.L., Bennink, J.R. and Moss, B. A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 1994, **12**, 1032–1040

11 Earl, P.L. and Moss, B. Generation of recombinant vaccinia viruses. In: *Current Protocols in Molecular Biology* (Eds Ausubel F.M., *et al.*). Greene Publishing Associates/Wiley Interscience, New York, 1991, pp. 16.17.1–16.17.16

12 Coelingh, K.V.W., Winter, C.C., Murphy, B.R. *et al.* Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. *J. Virol.* 1986, **60**, 90–96

13 Prince, G.A., Jenson, A.B., Hemming, V.G. *et al.* Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. *J. Virol.* 1986, **57**, 721–728

14 Rosel, J.L., Earl, P.L., Weir, J.P. and Moss, B. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment. *J. Virol.* 1986, **60**, 436–439

15 Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R.G. and Paoletti, E. Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine* 1988, **6**, 504–508

16 Guo, P., Goebel, S., Davis, S. *et al.* Expression in recombinant vaccinia virus of the equine herpesvirus 1 gene encoding glycoprotein gp13 and protection of immunized animals. *J. Virol.* 1989, **63**, 4189–4198

17 Perkus, M.E., Limbach, K. and Paoletti, E. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. *J. Virol.* 1989, **63**, 3829–3836

18 Davison, A.J. and Moss, B. The structure of vaccinia virus late promoters. *J. molec. Biol.* 1989, **210**, 771–784

19 Ebata, S.N., Cote, M.-J., Kang, C.Y. and Dimock, K. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. *Virology* 1991, **183**, 437–441

20 Moscona, A. and Peluso, R.W. Fusion properties of cells persistently infected with human parainfluenza virus type 3: participation of hemagglutinin-neuraminidase in membrane fusion. *J. Virol.* 1991, **65**, 2773–2777

21 Hu, X., Ray, R. and Compans, R.W. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. *J. Virol.* 1992, **66**, 1528–1534

22 Redfield, R.R., Wright, D.C., James, W.D., Jones, T.S., Brown, C. and Burke, D.S. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *N. Engl. J. Med.* 1987, **316**, 673–676

23 Buller, R.M.L., Smith, G.L., Cremer, K., Notkins, A.L. and Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* 1985, **317**, 813–815

24 Buller, R.M., Chakrabarti, S., Cooper, J.A., Twardzik, D.R. and Moss, B. Deletion of the vaccinia virus growth factor gene reduces virus virulence. *J. Virol.* 1988, **62**, 866–877

25 Tartaglia, J., Perkus, M.E., Taylor, J. *et al.* NYVAC—a highly attenuated strain of vaccinia virus. *Virology* 1992, **188**, 217–232

26 Taylor, J., Weinberg, R., Tartaglia, J. *et al.* Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. *Virology* 1987, **187**, 321–328

27 Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H.C., Schäfer, H. and Holzner, A. MVA-stufenimpfung gegen pocken. Klinische erprobung des attenuierten pocken-lebendimpfstoffes, stamm MVA. *Dtsch. Med. Wschr.* 1974, **99**, 2386–2392

28 Coupar, B.E.H., Andrew, M.E., Both, G.W. and Boyle, D.B. Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. *Eur. J. Immun.* 1986, **16**, 1479–1487

29 Townsend, A., Bastin, J., Gould, K. *et al.* Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. exp. Med.* 1988, **168**, 1211–1224

30 Small, P.A., Smith, G.L. and Moss, B. Intranasal vaccination with a recombinant vaccinia virus containing influenza hemag-

glutinin prevents both influenza virus pneumonia and nasal infection: intradermal vaccination prevents only viral pneumonia. In: *Vaccines 85* (Eds Lerner, R.A., Chanock, R.M. and Brown, F.). Cold Spring Harbor Laboratory, Cold Spring Harbor, 1985, pp. 175–176

31 Kanesaki, T., Murphy, B.R., Collins, P.L. and Ogra, P.L. Effectiveness of enteric immunization in the development of secretory immunoglobulin A response and the outcome of infection with respiratory syncytial virus. *J. Virol.* 1991, **65**, 657–663

32 Nussbaum, O., Broder, C.C. and Berger, E.A. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. *J. Virol.* 1994, **68**, 5411–5422

33 Chakrabarti, S., Brechling, K. and Moss, B. Vaccinia virus expression vector: coexpression of B-galactosidase provides visual screening of recombinant virus plaques. *Molec. Cell. Biol.* 1985, **5**, 3403–3409

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Host Range and Cytopathogenicity of the Highly Attenuated MVA Strain of Vaccinia Virus: Propagation and Generation of Recombinant Viruses in a Nonhuman Mammalian Cell Line

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Modified vaccinia virus Ankara (MVA), attenuated by over 500 passages in primary chick embyro fibroblasts (CEF), is presently being used as a safe expression vector. We compared the host ranges of MVA and the parental Ankara strain in CEF and 15 permanent cell lines. The cells could be grouped into three categories: permissive, semipermissive, and nonpermissive. For MVA, the permissive category consisted of primary CEF, a quail cell line derived from QT6, and the Syrian hamster cell line BHK-21. Only in BHK-21 cells did the virus yield approach that occurring in primary CEF. The semipermissive category included two African green monkey cell lines: BS-C-1 and CV-1. The nonpermissive category for MVA consisted of three human cell lines HeLa, 293, and SW 839; one rhesus monkey cell line FRhK-4; two Chinese hamster cell lines CHO and CHL; one pig cell line PK(15); and three rabbit cell lines RK13, RAB-9, and SIRC. The grouping for MVA with a restored K1L host range gene was similar except for the inclusion of RK13 cells among permissive lines. The grouping for the Ankara strain, however, was quite different with more permissive and semipermissive cell lines. Nevertheless, in cells that were permissive for MVA, the virus replicated to higher levels than Ankara, consistent with both positive and negative growth elements associated with the adaptation of MVA. The cell lines were also characterized according to their susceptibilty to MVA-induced cytopathic effects, expression of a late promoter regulated reporter gene by an MVA recombinant, and stage at which virion morphogenesis was blocked. Finally, the permissive BHK-21 cell line was shown to be competent for constructing and propagating recombinant MVA, providing an alternative to primary CEF.

INTRODUCTION

Modified vaccinia virus Ankara (MVA), a highly attenuated strain of vaccinia virus (VV), was developed as a safe vaccine for smallpox prior to the eradication of that disease. MVA was derived from the VV Ankara strain by over 500 passages in primary chick embryo fibroblasts (CEF), after which it could no longer replicate, or replicated very inefficiently, in a variety of mammalian cell lines (Mayr *et al.*, 1975, 1978). MVA is nonpathogenic in animals, including suckling and irradiated mice and primates (Hochstein-Mintzel *et al.*, 1972; Mayr *et al.*, 1978). In addition, no serious complications were reported when MVA was administered as a smallpox vaccine to over 100,000 humans (Mahnel and Mayr, 1994; Stickl *et al.*, 1974). In the United States, Biosafety level 2 containment and smallpox vaccination at 10-year intervals have been recommended for laboratory studies with standard VV strains (Katz and Broome, 1991). MVA, however, was recently assigned Biosafety level 1 status without a vaccination requirement by the National Institutes of Health intramural biosafety committee.

The genetic basis for the host range restriction of MVA is not yet understood. At least four orthopoxvirus host-range genes have been described: CHO*hr* (Gillard *et al.*, 1985), C7L (Oguiura *et al.*, 1993; Perkus *et al.*, 1990), K1L (Perkus *et al.*, 1990), and E3L (Beattie *et al.*, 1996; Chang *et al.*, 1995). The homolog of the cowpox CHO*hr* gene has been disrupted or deleted in all strains of VV and an intact gene is required for replication of VV in Chinese hamster ovary (CHO) cells. Expression of either CHO*hr*, K1L, or C7L allows VV replication in human MRC-5 and pig kidney PK(15) cells and either K1L or CHO*hr* permits replication in rabbit kidney RK13 cells (Perkus *et al.*, 1990). The E3L gene is required for VV replication in Vero and HeLa cells (Beattie *et al.*, 1996; Chang *et al.*, 1995). With the exception of the E3L gene, which codes for a double-stranded RNA binding protein (Chang *et al.*, 1992), the functions of the host-range genes are unknown. An examination of the MVA genome showed that approximately 15% of the parental DNA, including most of the K1L gene, had been deleted (Altenburger *et al.*, 1989; Meyer *et al.*, 1991). However, replacement of the K1L gene extended the host range of MVA only to RK13 cells. The functional status of other orthopoxvirus host range genes in MVA is still unknown.

The host range restriction of MVA is unique in several respects. First, the restriction is broader than that of other host range mutants: efficient replication has been described only in primary CEFs. Second, the block in nonpermissive cells occurs at a late stage of the replication cycle. Biochemical and electron microscopic analyses of HeLa cells infected with MVA indicated that viral late

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gene expression occurs but that virion morphogenesis is interrupted (Sutter and Moss, 1992). In contrast, viral protein synthesis is inhibited soon after nonpermissive infections with mutants of other known host range genes (Chang *et al.*, 1995; Gillard, 1985, Njayou *et al.*, 1982; Ramsey-Ewing and Moss, 1995, 1996). The unimpaired viral protein synthesis, even in nonpermissive human cells, is an important property of MVA that accounts for its usefulness as a safe yet efficient expression vector (Sutter and Moss, 1992).

Transfer plasmids for construction of stable MVA expression vectors have been developed (Antoine *et al.*, 1996; Carroll and Moss, 1995; Scheiflinger *et al.*, 1996; Sutter and Moss, 1992; Wyatt *et al.*, 1996) and the bacteriophage T7 RNA polymerase gene has been integrated into the MVA genome for transient transfection studies (Sutter *et al.*, 1995; Wyatt *et al.*, 1995). Furthermore, animal experiments indicated that recombinant MVA provided protection against challenge with influenza virus (Bender *et al.*, 1996; Sutter *et al.*, 1994), simian immunodeficiency virus (Hirsch *et al.*, 1996), and parainfluenza virus type 3 (Wyatt *et al.*, 1996). Where comparisons were made, the immunogenicity of MVA recombinants was equal to or better than those of standard VV recombinants. In addition, MVA has been effective in a murine cancer immunotherapy model (Carroll *et al.*, 1997).

As the use of MVA vectors expanded, more cell lines were used to express recombinant proteins, and our experience and that of others indicated that the cytopathic effects (CPE) of MVA or MVA recombinants varied. In some mammalian cell lines, such as BHK-21 cells, the CPE seemed comparable to those of standard VV strains (J. K. Rose, G. Wertz, personal communications). Scientific questions pertaining to the host range of MVA, as well as practical ones concerning its use as an expression vector, motivated us to study MVA with regard to induction of CPE, replication, cell to cell spread, virus morphogenesis, and recombinant gene expression in additional mammalian and avian cell lines. For comparison, we also analyzed the host range properties of the parental Ankara strain of VV and the effects of a functional K1L gene on MVA host range restriction.

MATERIALS AND METHODS

Cells and viruses

Cell lines (Table 1) were grown under conditions suggested by the American Type Culture Collection (ATCC, Rockville, MD). CEF were prepared from 10-day-old embryos. For virus infections, CEF were used in the first passage although they are referred to as primary CEF in the text. The quail cell line QT35 (supplied by K. Anderson, USAMRIID, Frederick, MD) is a derivative of QT6 (ATCC) and was grown in E-199 medium containing 10% tryptose phosphate broth and 5% fetal bovine serum (FBS). MVA and the parental strain VV Ankara (provided by A. Mayr, Veterinary Faculty, University of Munich) and MVA/K1L, an MVA recombinant containing a functional K1L gene (Meyer *et al.*, 1991), were propagated in CEF cells and purified by centrifugation through a 36% sucrose cushion (Earl *et al.*, 1991). Virus stocks were titered on CEF by immunostaining as described below. MVA *lac*Z (Sutter and Moss, 1992) and WR *lac*Z (Carroll and Moss, unpublished data) are recombinant VV derived from MVA and WR strains, respectively. Both viruses contain the *Escherichia coli lac*Z gene encoding β-galactosidase (GAL) under the regulation of the VV 11K late promoter (Bertholet *et al.*, 1985).

Immunostaining

MVA does not form clear plaques on cell monolayers and therefore foci were visualized by immunostaining. Briefly, cells were fixed with a 1:1 solution of methanol:acetone for 2 min, washed with phosphate buffered saline (PBS), and then incubated for 1 h at room temperature with rabbit polyclonal VV antiserum diluted 1:2000 with PBS containing 2% FBS. After washing twice with PBS, cells were incubated for 1 h at room temperature with Protein A conjugated to horseradish peroxidase (HRP, Boerhinger) diluted to 1:1500 in PBS containing 2% FBS. Cells were washed twice in PBS and incubated up to 30 min with substrate solution prepared by adding 10 μl of 30% H_2O_2 and 0.2 ml of an ethanol solution saturated with dianisidine (Sigma) to 10 ml of PBS. A brown stain signified VV proteins. Double immunostaining, to visualize recombinant protein synthesis and MVA, was done as follows: monolayers were treated as above except that the 1:500 dilution of the primary antibody applied was against GAL or β-glucuronidase (GUS). Protein A–HRP and substrate were then applied as described above. GAL or GUS positive foci were enumerated and the anti-VV sera was then used to identify all MVA foci.

Virus replication

For analysis of virus replication and spread, confluent monolayers in Costar six-well tissue culture dishes were infected at a multiplicity of infection (m.o.i.) of approximately 0.05 using a total of 5×10^4 plaque forming units (PFU) in 1 ml of medium containing 2% FBS. After 45 min at 37°, cells were washed once with medium containing 2% FBS and incubated with fresh medium at 37°. Cells and supernatant were harvested at 0, 24, 48, and 72 h after absorption. For single step growth curves, cells were infected with an m.o.i. of 5. After freeze–thawing thrice and brief sonication, samples were assayed in duplicate on monolayers of CEF. Infected cell foci were visualized by immunostaining after 24 h.

Measurement of GAL

Cells (approximately 2×10^5) were infected at an m.o.i. of 5 at 37°. After 1 h, monolayers were washed with PBS and incubated with medium containing 2% FBS. Cells were washed and harvested in Promega reporter lysis

TABLE 1

Spread and Replication of MVA and Ankara after Low Multiplicity infections[a]

Cell line	ATCC code	Species	Organ	Morphology	Virus spread[b]		Virus replication[c]			
					MVA	Ankara	MVA		Ankara	
CEF	Primary	Chick embryo	Assorted	Fibroblast	+++	+++	4,200 ± 200	(P)	240 ± 120	(P)
QT35[d]	CRL-1708	Quail embryo	Assorted	Fibroblast	ND	ND	69.8 ± 30	(P)	12.0 ± 8	(SP)
BHK-21	CCL-10	Hamster, Syrian	Kidney	Fibroblast	+++	+++	680 ± 240	(P)	320 ± 200	(P)
CHO	CCL-61	Hamster, Chinese	Ovary	Epithelial	−	−	<0.001	(NP)	<0.001	(NP)
CHL	CRL-1935	Hamster, Chinese	Lung	Fibroblast	ND	ND	0.24	(NP)	0.12	(NP)
BS-C-1	CCL-26	Monkey, African green	Kidney	Epithelial	++	+++	23.6 ± 0.4	(SP)	82.0 ± 38	(P)
CV-1	CCL-70	Monkey, African green	Kidney	Fibroblast	++	+++	9.60 ± 2.4	(SP)	30.0 ± 14	(P)
FRhK-4	CRL-1688	Monkey, Rhesus	Kidney	Fibroblast	ND	ND	0.8	(NP)	6.0	(SP)
293	CRL-1573	Human	Kidney	Epithelial	+	+++	0.03 ± 0.004	(NP)	49.0 ± 27	(P)
HeLa	CCL-2	Human	Cervix	Epithelial	+	+++	0.07 ± 0.06	(NP)	12.5 ± 12	(SP)
SW 839	HTB-49	Human	Kidney	Fibroblast	ND	ND	0.003	(NP)	0.04	(NP)
PK(15)	CCL-33	Pig	Kidney	Epithelial	−	+++	0.004	(NP)	44.0	(P)
MDCK	CCL-34	Canine	Kidney	Epithelial	ND	ND	2.3 ± 0.1	(SP)	106 ± 18	(P)
RK13	CCL-37	Rabbit	Kidney	Epithelial	−	+++	0.001	(NP)	160	(P)
RAB-9	CRL-1414	Rabbit	Skin	Fibroblast	+	+++	0.98 ± 0.58	(NP)	11.0 ± 7	(SP)
SIRC	CCL-60	Rabbit	Cornea	Fibroblast	+	+++	0.03	(NP)	136	(P)

[a] m.o.i. of 0.05.

[b] Virus spread as visualized by immunostaining after 72 h. −, No stained cells; +, foci of 1 to 4 stained cells; ++, foci of 5 to 25 stained cells; +++, foci of >25 stained cells; ND, not determined.

[c] Virus replication (fold increase in virus titer) determined by dividing the virus yield at 72 h by the input titer of 2.5×10^5. Letters in parentheses refer to permissive, semipermissive, and nonpermissive for virus replication: NP, <1-fold increase; SP, 1- to 25-fold increase; P, >25-fold increase.

[d] QT35 cells supplied by K. Anderson were derived from QT6 cells. The ATCC code refers to QT6 cells.

buffer. GAL activity was determined using a Promega (Madison, WI) enzyme assay kit. Standard curves were made to determine GAL activity units.

Electron microscopic analysis

Confluent cell monolayers were infected at a m.o.i. of 10. The virus was allowed to adsorb for 1 h at 37° and the cells were then washed and incubated at 37° for an additional 24 h. Cells were fixed in 2% glutaraldehyde and processsed for transmission electron microscopy as previously described (Wolffe *et al.*, 1996), except that sections were first stained with 7% uranyl acetate (Electron Microscopy Sciences, Fort Washington, PA) in 50% ethanol for 20 min and then with bismuth subnitrate for 5 min as described (Ainsworth and Karnovsky, 1972).

Recombinant virus construction

Monolayers of CEF or BHK-21 cells, in six-well tissue culture dishes, were infected with MVA at an m.o.i. of 0.1. After 1 h at 37°, cells were washed twice and overlayed with 1 ml of Optimem (Gibco BRL). Approximately 3 μg of plasmid DNA was diluted with sterile water to 25 μl and mixed with 15 μg of lipofectin (Gibco BRL) previously diluted to a final volume of 25 μl. The DNA/lipofectin mixture was incubated at room temperature for 10 min and then added dropwise to the infected cells. After a 4-h absorption, the Optimem was replaced with 1 ml of medium containing 2% FBS. Cells were harvested after an additional 40 h, frozen and thawed thrice, sonicated, and inoculated on CEF or BHK-21 cells. Recombinant virus plaques expressing GAL or GUS were identified using the substrates X-gal (Gold BioTechnology, St. Louis, MO) or X-glu (Clonetech Laboratories, Palo Alto, CA), respectively (Carroll and Moss, 1995). After 1 h absorption, cells were overlayed with 3 ml of plaque medium containing 2% FBS and 1% low melting point (LMP) agarose (Gibco BRL). Cells were incubated for approximately 40 h and overlayed with 2 ml of plaque medium containing the relevant substrate. A blue color was evident within 6 to 18 h.

RESULTS

Cell to cell spread of MVA and VV Ankara

As a sensitive measure of the host-range properties of MVA and the parental Ankara strain of VV, we infected primary CEF and 10 different cell lines with an m.o.i. of 0.01 and measured virus spread. As spread might occur in the absence of obvious CPE, rabbit polyclonal anti-VV serum was used to identify cells expressing MVA proteins. In permissive CEF, MVA produced clearly stained foci of approximately 100 cells at 24 h after infection (Fig. 1). The stained cells maintained their spindle shape with little or no evidence of CPE until several days later. Foci formed by the parental VV Ankara strain in CEF were slightly larger, with gaps or holes in their centers, after 1 day (Fig. 1).

Remarkably, the viral protein staining pattern observed in MVA infected Syrian baby hamster kidney BHK-21 cells

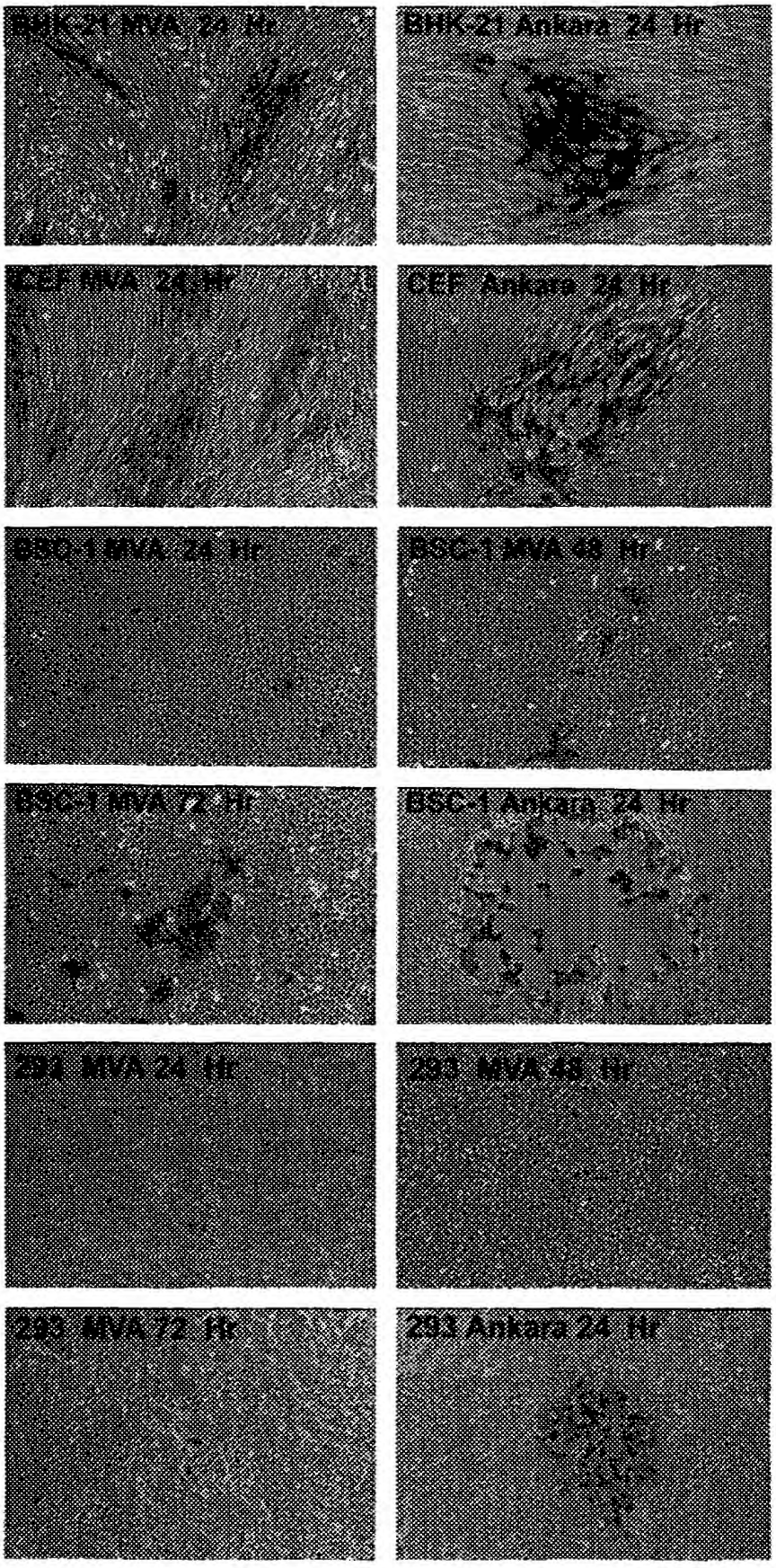

FIG. 1. Cell-to-cell spread of MVA and VV Ankara. The indicated cells were infected with an m.o.i. of 0.01 and then fixed and immunostained with anti-VV antibody at 24, 48, or 72 h. The panels show representative fields at approximately 200× magnification.

was similar to that in CEF (Fig. 1), suggesting that these mammalian cells were able to support replication and spread of this virus. The sizes of the foci were similar to those seen in CEF cells and the entire monolayer showed cytopathic effects after longer times. No other mammalian cell line examined supported such extensive spread of MVA. The greater cytopathic effects of VV Ankara, compared to MVA, could be seen at 24 h after infection of BHK-21 cells.

Other mammalian cell lines, exemplified by BS-C-1 (Fig. 1) and CV-1 (photograph not shown), enabled limited spread of MVA. At 24 h, foci containing 2 to 5 cells were frequently observed. The size of these foci steadily increased to about 20 cells by 72 h (Fig. 1). To determine if the apparent spread of MVA in BS-C-1 cells was merely due to diffusion of MVA gene products, we specifically inhibited virus maturation with 100 μg/ml of rifampicin (Moss *et al.*, 1969). After a 72-h incubation, intense immunostaining occurred mainly in single cells with occasional weak staining of immediately adjacent cells (data not shown). The effect of rifampicin suggested that foci forming in the absence of drug were due to spread of mature infectious virions.

Several MVA inoculated cell lines, exemplified by human 293 (Fig. 1) and HeLa (photograph not shown), developed predominantly single stained cells with occasional foci of less than five stained cells after a 72-h incubation. A pattern of almost exclusively single staining cells had previously been noted with mouse L-929 cells (Sutter *et al.*, 1994). Another category of cells, represented by CHO and RK13, did not show any evidence of MVA protein production when immunostained (photograph not shown). Control experiments, carried out in permissive cell lines infected in the presence of the DNA replication inhibitor AraC, indicated that viral late protein synthesis was required for detectable immunostaining. Therefore, the block in CHO and RK13 cells occurred at a stage before viral late gene expression. These results are consistent with the stages at which standard VV strains lacking the CHO*hr* or K1L gene are blocked in CHO cells and RK13 cells, respectively (Ramsey-Ewing and Moss, 1995, 1996).

A summary of the data regarding MVA spread and a description of the 11 cell types examined are in Table 1.

Replication of MVA, MVA/K1L, and VV Ankara under multistep growth conditions

The ability of VV Ankara and MVA to replicate and spread was further investigated and quantitated in primary CEF and 15 different cell lines infected at a m.o.i. of 0.05. We also examined the replication of a previously decribed (Meyer *et al.*, 1991) recombinant MVA with a restored K1L gene (referred to as MVA/K1L). At 0, 24, 48, and 72 h after infection, the cells were harvested and titered in CEF. As previously noted (Meyer *et al.*, 1991), MVA replicated to a higher titer in CEF than the parental

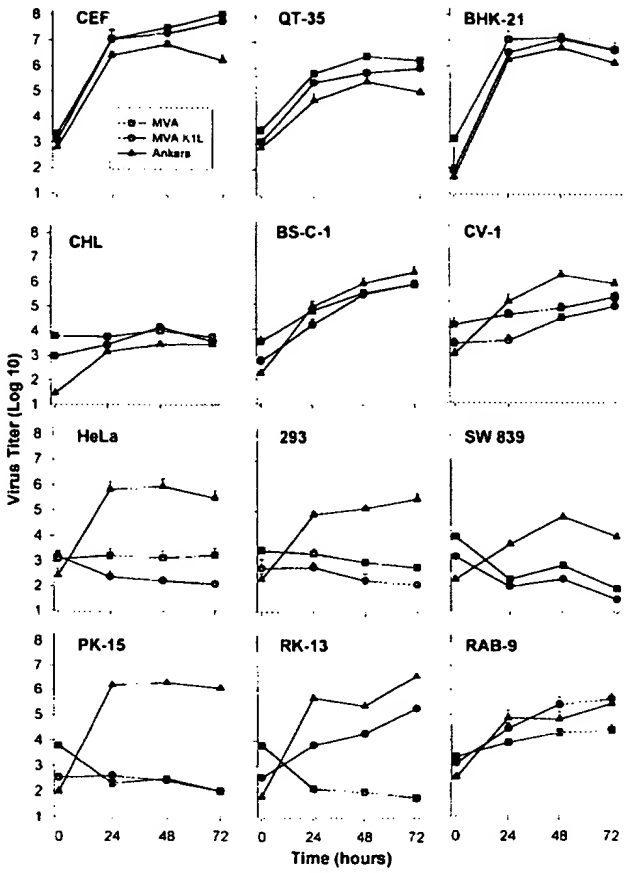

FIG. 2. Replication of MVA, MVA/K1L, and VV Ankara in selected cell lines. Cells were infected with an m.o.i. of 0.05 and after adsorption and 24, 48, and 72 h later, the medium and cells were harvested together. Virus yields were determined by infecting CEF with serial dilutions and counting immunostained cell foci. The titers represent the average values of two independent experiments, except for SW 839, PK(15), and RK13, which were done once. Standard errors are indicated by bars.

VV Ankara strain (Fig. 2). Restoration of the K1L gene, however, had no significant effect on MVA replication. One avian cell line tested, quail QT35, was permissive for both MVA and VV Ankara but the yields were 1 to 2 logs lower than with CEF (Fig. 2).

Three hamster cell lines were tested. The yield of MVA in Syrian hamster BHK-21 cells was similar to that in primary CEF at 24 h, but then plateaued so that the 72-h yield was slghtly less than in CEF (Fig. 2). The presence or absence of a functional K1L gene made no difference to MVA replication in BHK-21 cells. The yield of VV Ankara in BHK-21 cells was slightly lower than that of MVA. The two Chinese hamster cell lines tested, CHO (Drillien *et al.*, 1978) and CHL (Ramsey-Ewing, personal communication), are not permissive for standard laboratory strains of VV so their failure to support replication of MVA or MVA/K1L was predictable (Fig. 2; Table 1).

Three monkey cell lines were evaluated. Significant but low increases in MVA titers were obtained in the African green monkey kidney lines, BS-C-1 and CV-1 (Fig.

2). The K1L gene did not increase the production of MVA in these cells. However, VV Ankara grew better than MVA in BS-C-1 and CV-1 cells. Replication of MVA was poor in rhesus FRhK-4 kidney cells (data shown in Table 1).

In all three human cell lines tested, HeLa, 293, and SW 839, the titers of MVA remained constant or decreased after the adsorption period whereas the parental VV Ankara strain replicated (Fig.2). Here too, a functional K1L gene was not beneficial for MVA replication.

Three rabbit cell lines were evaluated. As previously described, MVA did not replicate in rabbit kidney (RK13) cells, whereas both MVA/K1L and Ankara did (Fig. 2). MVA/K1L and VV Ankara also grew better than MVA in rabbit skin (RAB-9) cells but this cell line was inefficient even for those strains (Fig. 2). Rabbit cornea (SIRC) cells were permissive for Ankara but not MVA (data shown in Table 1). K1L enhanced MVA replication in SIRC cells to a small extent (data not shown).

The 72-h virus yields were divided by the amount of input virus (2.5×10^4) and the ratios are listed in Table 1. Higher ratios would have been obtained if the yields were divided by the virus titers determined after the absorption period (Fig. 2). However, that would have resulted in greater variability especially as the postabsorption VV Ankara titers were usually 1 log lower than those of either MVA or MVA/K1L, though the input titers were similar. Cell lines were categorized using the following criteria: nonpermissive allowing a <1-fold replication, semipermissive allowing 1- to 25-fold replication, and permissive allowing >25-fold replication. These categories were in agreement with the more qualitative immunocytological assessment of virus spread (Table 1). Two cell lines fell into the permissive group for MVA: BHK-21 and QT35 cells. Of these, BHK-21 cells consistently gave the higher yield. BS-C-1, CV-1 and MDCK cells were in the semipermissive group and the others including all of the human cells were nonpermissive. The patterns of permissiveness for VV Ankara and MVA were quite different (Table 1). Thus, for VV Ankara, the BHK-21, BS-C-1, CV-1, 293, PK(15), MDCK, RK13, and SIRC cell lines were permissive; QT35, FRhK4, HeLa, and RAB-9 cell lines were semipermissive; and only CHO and CHL cells were nonpermissive. SW 839 cells also scored as nonpermissive, although slight replication was suggested by growth curves (data not shown).

Replication and CPE in synchronized MVA infections

Under the low multiplicity, multistep growth conditions in the preceding section, both the formation and spread of infectious virus were important. There are many VV mutants in which normal intracellular levels of infectious virus are formed but plaque size and cell to cell spread are severely reduced (Blasco and Moss, 1991; Dallo *et al.*, 1987; Duncan and Smith, 1992; Martinez-Pomares *et al.*, 1993). Under these circumstances, very different virus yields are obtained when multistep and single step

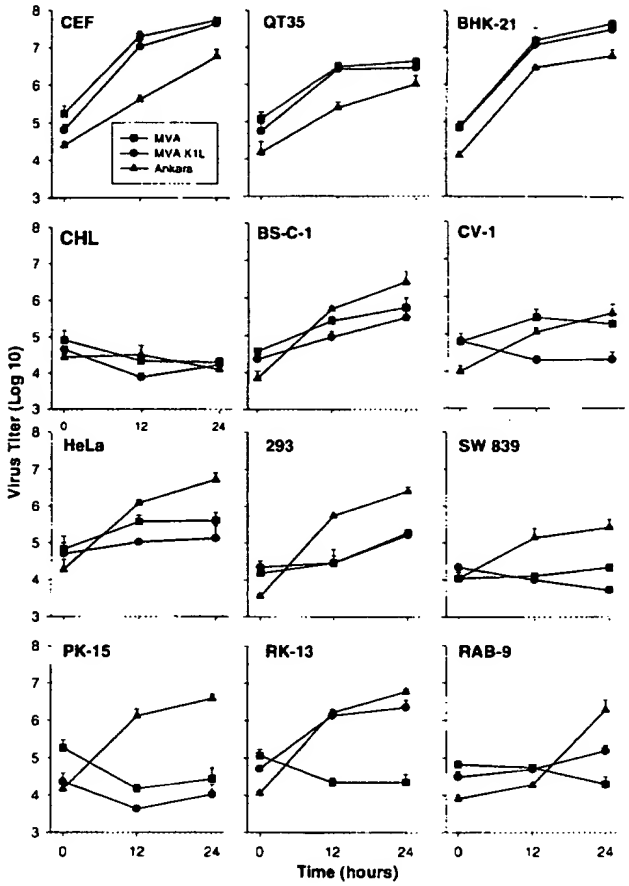

FIG. 3. Synchronous infection of selected cells by MVA, MVA/K1L, and VV Ankara. Cells were infected with an m.o.i. of 5 and the medium and cells were harvested together and the virus yields were determined as in the legend to Fig. 2. The titers represent the average of values from two independent experiments. Standard errors are indicated by bars.

growth experiments are compared. We therefore determined the virus titers following synchronous infections at an m.o.i. of 5.

As expected, cells that enabled multistep growth of MVA produced significant yields of virus under one-step growth conditions. Thus, similar high yields of MVA were obtained in primary CEF and continuous BHK-21 cells and almost a log lower yields in QT35 cells. (Fig. 3). Only these three cell types produced more than 1 PFU of MVA per cell (Table 2).

Some increase in MVA titers were detected in BS-C-1 and CV-1 cells (Fig. 3). When comparing MVA titers between the adsorption period and 24 h, there was a slight increase in the human HeLa and 293 cell lines, though this was at least a log lower than for VV Ankara. Replication of MVA was still lower in MDCK cells; under multistep conditions these cells barely scored as semipermissive. Declining growth curves were seen in all other cells tested.

Restoration of the MVA K1L gene had a markedly positive effect only in RK13 cells. VV Ankara replicated to some extent in all cell lines except CHO and CHL. How-

TABLE 2

Cytopathic Effects and Replication of MVA and Ankara after Synchronous Infections[a]

Cell line	MVA CPE[b] 12 h	MVA CPE 24 h	MVA PFU/cell	MVA/K1L CPE 12 h	MVA/K1L CPE 24 h	MVA/K1L PFU/cell	Ankara CPE 12 h	Ankara CPE 24 h	Ankara PFU/cell, 24 h
CEF	+	+++	55.30 ± 6	+/++	+++	43 ± 4.7	+++	++++	6.0 ± 3.2
QT35	+	+++	4.85 ± 0.55	+/++	+++	5.50 ± 0.8	++++	++++	1.5 ± 0.5
BHK-21	+	++	59.0 ± 14	+	++	37 ± 3.7	++	+++	9.7 ± 4.7
CHO	+	+	0.01 ± <0.01	+	+	0.01 ± <0.01	+	+/++	<0.01 ± <0.01
CHL	++	+++	0.01 ± <0.01	++	+++	0.01 ± <0.01	+++	++++	0.01 ± <0.01
BS-C-1	−	+	1.00 ± 0.7	−	+	0.57 ± 0.27	++++	++++	4.6 ± 3.75
CV-1	+	+	0.27 ± 0.07	+	+	0.1 ± 0.03	++	++++	1.5 ± 1.1
FRhK-4	+/++	++	0.27 ± 0.07	++	++	0.26 ± 0.07	+++	++++	0.55 ± 0.45
293	+++	++++	0.25 ± 0.11	+++	++++	0.21 ± 0.08	ND	++++	4.2 ± 2.8
HeLa	+	++++	0.56 ± 0.2	+	++++	0.22 ± 0.12	+	++++	7.0 ± 2
SW 839	+	+++	0.17 ± 0.13	+	+++	0.08 ± 0.07	+++	++++	0.63 ± 0.33
PK(15)[c]	−	+	0.04 ± 0.02	−	+	0.02 ± 0	+/++	++	4.17 ± 0.83
MDCK	ND	ND	0.18	ND	ND	0.01	ND	ND	1.1
RK13	+	+/++	0.07 ± 0.01	+	+/++	3.42 ± 2.42	+++	++++	7.67 ± 0.67
RAB-9[d]	−/+	+	0.07 ± 0.03	−/+	+	0.62 ± 0.09	−	+/++	5.45 ± 3.55
SIRC	ND	ND	0.03 ± 0	ND	ND	0.08 ± 0	ND	ND	0.2 ± 0

[a] m.o.i. of 5.0.

[b] CPE was categorized by the following criteria: no difference from control, −; <25% CPE, +; 25 to 50% CPE, ++; >50 to 75% CPE +++; >75 to 100% CPE or high level cell detachment, ++++.

[c] CPE was + at 72 h and +++ at 144 h.

[d] CPE was + at 72 h and +++/++++ at 144 h.

ever, the yields were lower than MVA in CEF, QT35, and BHK-21 cells and higher in the others.

In parallel with the one-step growth curves, we examined the cells for CPE. Those cell lines that were permissive for MVA replication generally showed CPE, although this was delayed somewhat compared to VV Ankara (Table 2). Some cell lines that were nonpermissive for MVA had quite severe CPE by 24 h (e.g., 293, HeLa, CHL), while with others the CPE was delayed (e.g., BS-C-1, CV-1, PK(15), and RAB-9); PK(15) and RAB-9 cells exhibited only minor CPE even after 72 h.

Virus morphogenesis in permissive, semipermissive and nonpermissive cells

Sutter and Moss (1992) reported that only stages up to and including immature, spherical, enveloped viral particles were seen by electron microscopic examination of HeLa cells infected with MVA, whereas mature-looking particles were abundant in CEF. We extended this analysis to MVA or Ankara infected CEF, BHK-21, BS-C-1, and RAB-9 cells and repeated the analysis of HeLa cells.

As predicted, viral particles of all stages of maturation were evident in MVA infected BHK-21 cells (Figs. 4A, 4B) and CEF (not shown). The viral forms were indistinguishable from those of VV Ankara infected BHK-21 cells and CEF (not shown).

Mostly immature particles were seen in MVA-infected BS-C-1 cells, whereas mature particles were rare (Figs. 4C, 4D). In addition, some of the immature, spherical viral particles that formed in BS-C-1 cells contained dense nucleoid structures. Mature viral particles were abundant in BS-C-1 cells infected with VV Ankara (not shown).

As previously reported (Sutter and Moss, 1992), the vast majority of viral particles in MVA-infected HeLa cells were immature. In the present study, we were able to distinguish two types of spherical immature particles: the majority were typical, e.g., appearing in cross-section as a membrane enclosing granular material; others appeared dense and were frequently associated with cisternae (Fig. 4E). Some of the typical immature virions contained nucleoids (Fig. 4F), not noted previously in MVA-infected HeLa cells (Sutter and Moss, 1992). Remarkably, many of the dense immature particles appeared to be undergoing membrane wrapping and some were outside of the cell (Fig. 4F).

In RAB-9 cells infected with MVA, even immature viral particles were rare (not shown). Mature virions were detected in RAB-9 cells infected with VV Ankara, but the numbers were lower than in CEF or BS-C-1 cells.

Late promoter regulated reporter gene expression

VV gene expression is programmed so that early genes are expressed before DNA replication and intermediate and late ones successively afterward. As mentioned earlier, the ability to express late genes in nonpermissive cells is a distinguishing feature of MVA host

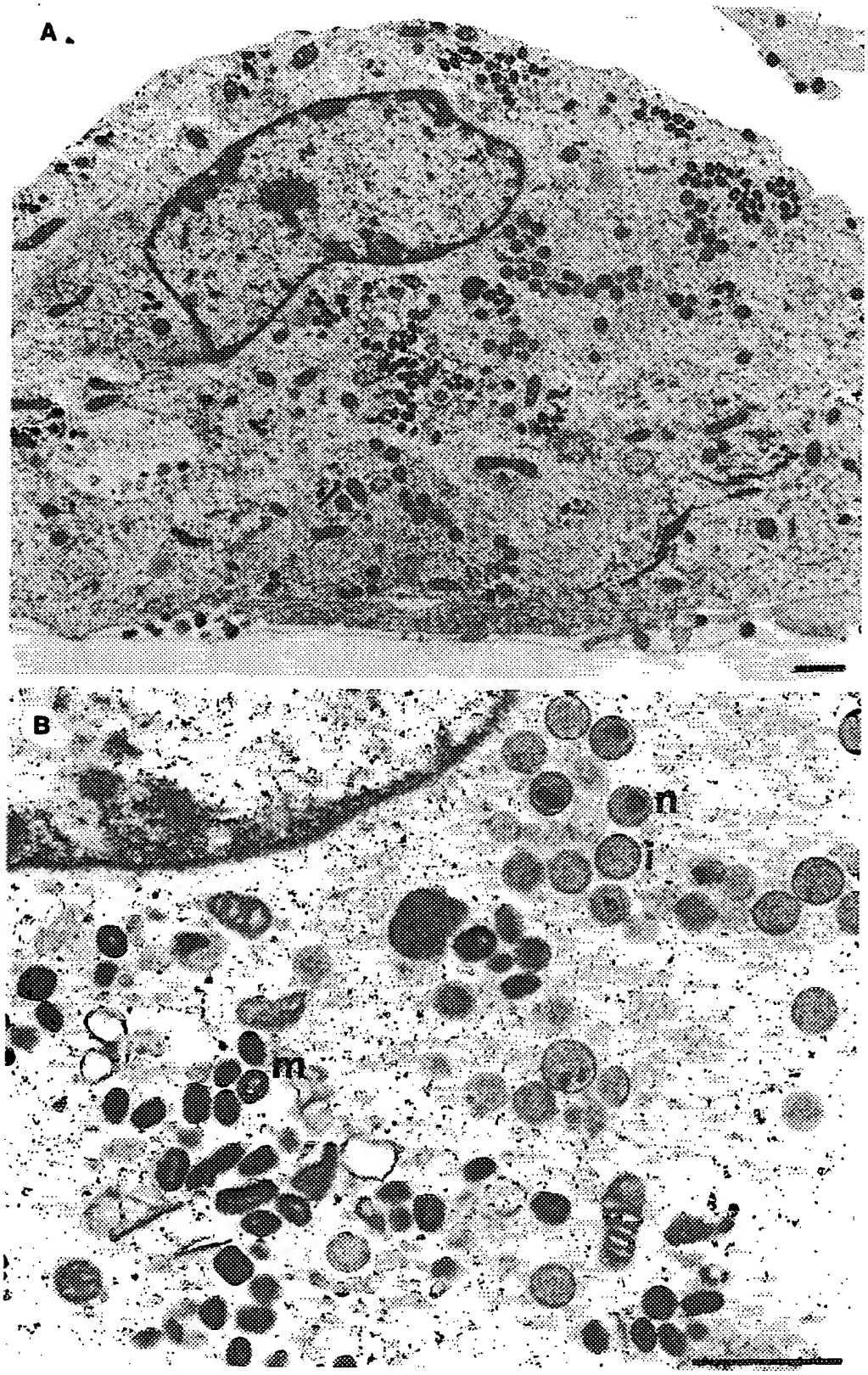

FIG. 4. Electron micrographs of ultrathin sections of BHK-21 (A, B), BS-C-1 (C, D), and HeLa (E, F, G) cells infected with MVA. i, typical immature virion; d, dense immature virion; m, brick-shaped mature virion; n, nucleoid; w, wrapping membrane. Bars, 1.0 μm.

restriction and enhances its value as an expression vector. We compared the expression of GAL regulated by the same 11K late promoter in MVA and WR recombinant viruses. The virus stocks had been purified by sedimentation through a 36% sucrose cushion, to remove contaminating GAL produced in the permissive cell line used for

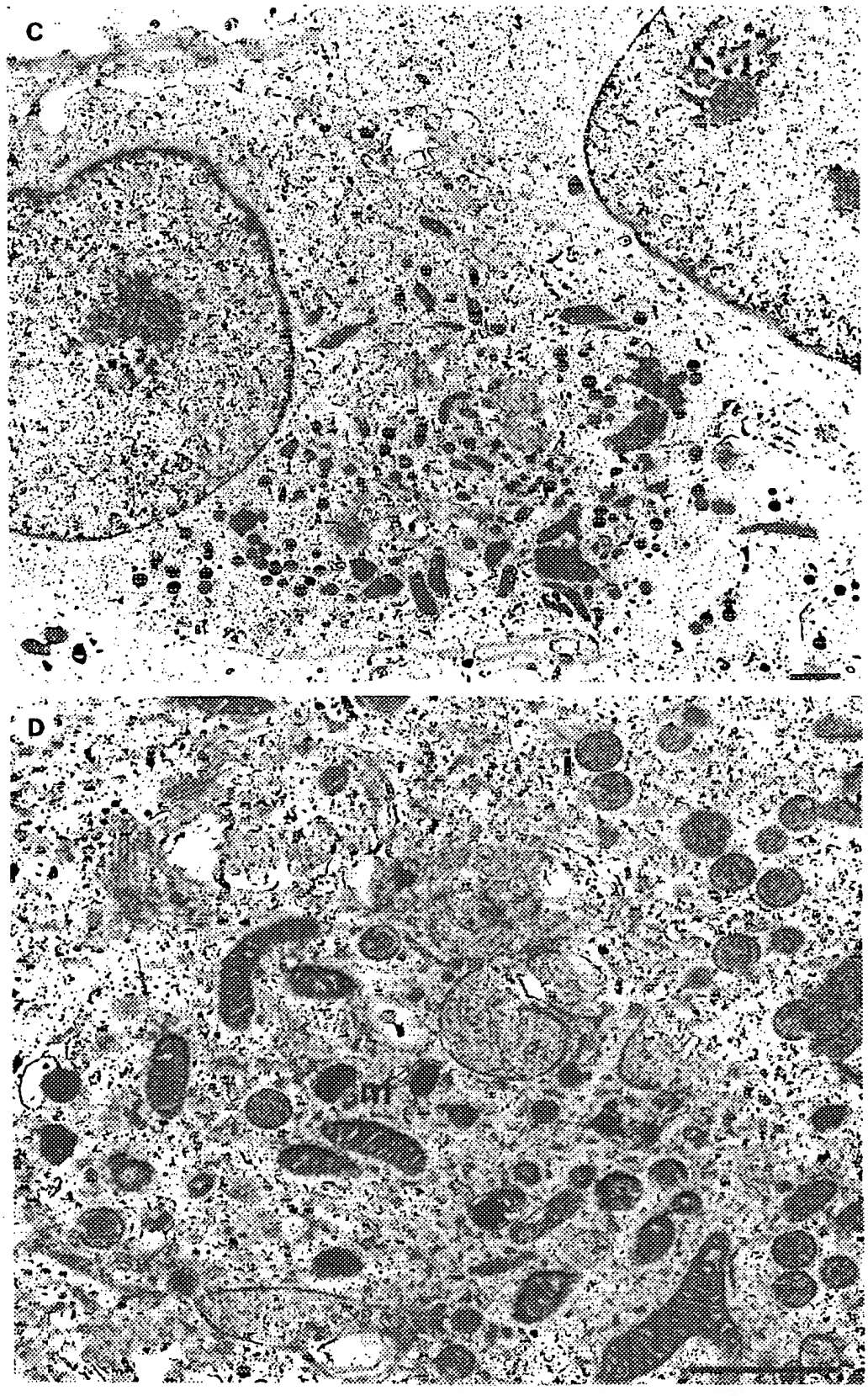

FIG. 4 — *Continued*

virus propagation. The highest levels of GAL were made in CEF infected with either MVA or WR recombinant viruses (Fig. 5). The next highest levels were made in BHK-21 cells by either of the recombinant viruses. In BS-C-1 and CV-1 cells, the WR recombinants produced several-fold more GAL than the MVA recombinants. The two cell

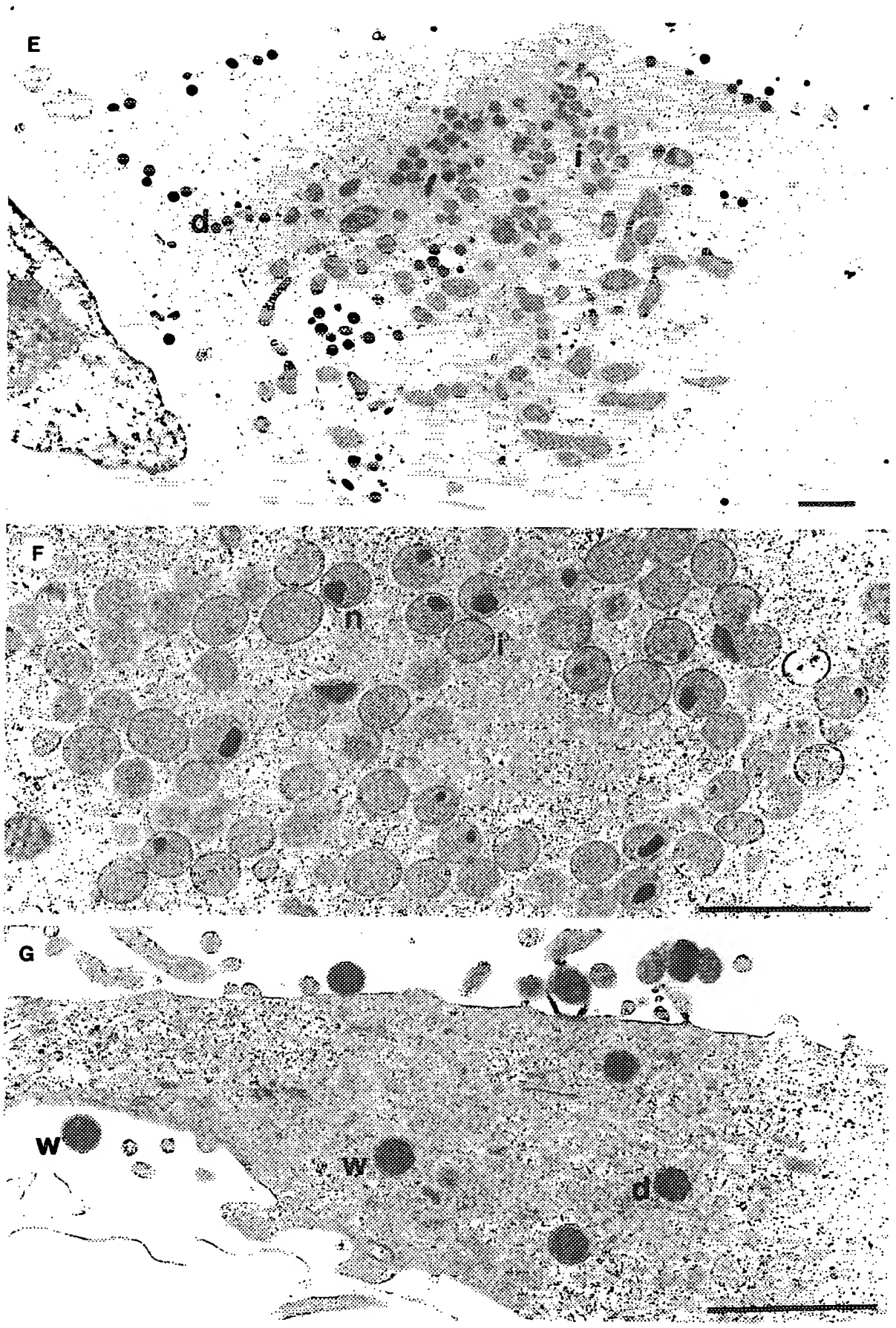

FIG. 4 — *Continued*

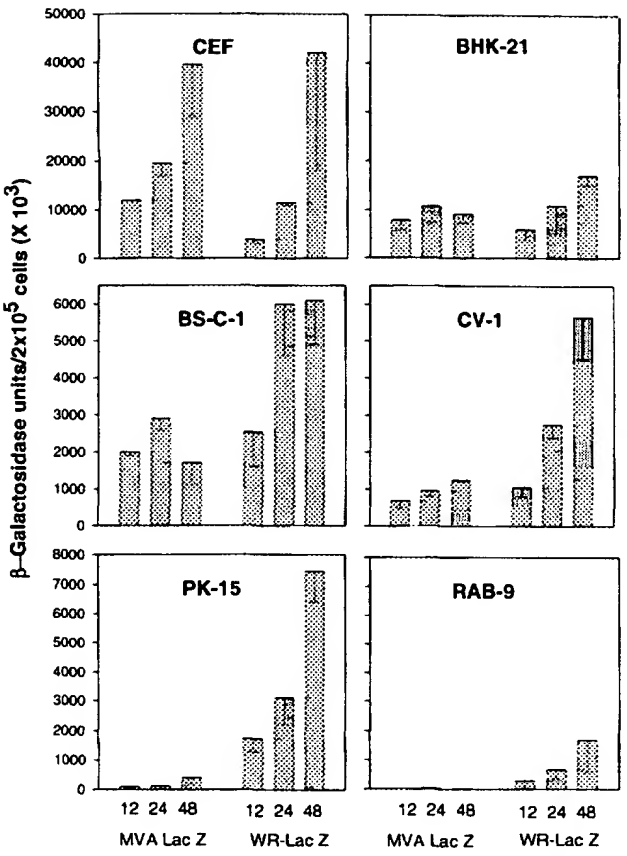

FIG. 5. Expression of *lacZ* under VV late promoter control by recombinant MVA and WR. MVA and WR recombinants containing the *E. coli lacZ* gene regulated by the VV 11K gene late promoter were used to infect cells at an m.o.i. of 5. The cells were harvested at 12, 24, and 48 h after infection and lysates were tested for GAL activity. GAL units per 2×10^5 cells were calculated from a standard curve. Standard error bars are shown.

lines that exhibited the least MVA-induced CPE, PK(15), and RAB-9 also produced the least GAL. Although the WR recombinant virus produced reasonable amounts of GAL in PK(15) cells, very little was made in RAB-9 cells.

Isolation of recombinant MVA in BHK-21 cells

The necessity of using primary CEF for the production of recombinant MVA hinders its widespread use as a safe alternative to fully replication competent VV strains such as WR. In an attempt to overcome this barrier, we compared the production of recombinant MVA in CEF and BHK-21 cells using transfer plasmids that introduced foreign genes into three different regions of the MVA genome (Table 3). Two of the three regions were sites of deletions that occurred during the multiple passages of MVA in CEF (Meyer *et al.*, 1991) and were chosen so that no additional viral genes would be interrupted. The third site was the *tk* locus, a site that is commonly used with replication competent VV strains such as WR. With transfer plasmid pMC03, the recombination efficiencies was similar in CEF and BHK-21 cells, with the detection of approximately 200 and 400 recombinants, respectively (Table 3). Approximately 500 recombinants were detected using the transfer plasmid pLW22 in BHK-21 cells. The results with transfer plasmid pMC1107GUS were similar: 200 recombinants in CEF and 300 in BHK-21 cells. In each case, the recombinant viruses were subsequently plaque purified three times and small stocks were prepared in CEF or BHK-21 cells. All plaques, produced by these stocks, stained with antibodies to the marker protein and to VV, confirming the purity and stability of the recombinant viruses in both CEF and BHK-21 cells.

DISCUSSION

VV has a remarkably broad host range and is capable of productively infecting avian as well as mammalian cells. In the case of MVA, repeated passaging in primary CEF resulted in an adaptation to CEF and diminished ability to replicate in most cell lines. Since the early passages of MVA were not preserved, we do not know whether the same or separate mutations accounted for the positive and negative cell-specific growth characteristics. The present study was undertaken to document further the host restriction of MVA and to facilitate its use as an expression vector. In addition to primary CEF, we infected 15 different cell lines with MVA and grouped them as permissive, semipermissive, and nonpermissive. Although the permissiveness of the quail cell line QT35 was not surprising in view of the avian adaptation of MVA and the permissiveness of CEF lines (Meyer *et al.*, 1991; cited in Sutter and Moss, 1992), the ability of MVA to replicate in a Syrian hamster cell line was remarkable. In fact, BHK-21 cells supported replication of MVA nearly as well as primary CEF and much better than the avian cell lines. Moreover, MVA replicates better in BHK-21 cells than the parental VV Ankara strain, indicating that the adaptation to CEF carried over to BHK-21 cells. Two African green monkey cell lines, BS-C-1 and CV-1, supported a low level of MVA replication, consistent with a previous report of MVA replication in MA 104 cells (Meyer *et al.*, 1991). Nevertheless, the three human cell lines tested here, as well as three additional ones tested by Meyer *et al.* (Meyer *et al.*, 1991), produced 1 PFU or less of MVA per cell and were classified as nonpermissive. Cells derived from a variety of other sources were also nonpermissive for MVA. There does not appear to be a recognizable feature that distinguishes cells that are permissive from those that are semi- or nonpermissive for MVA. Of the two permissive cell lines, one was of avian origin and the other was Syrian hamster derived and both were fibroblastic in morphology.

Host range restriction could result from inhibition of infectious virus formation or spread. Thus far, two types of mutations are known to prevent VV spread. One type reduces the amount of extracellular virus (Blasco and Moss, 1991; Engelstad and Smith, 1993; Rodriguez and Smith, 1990; Wolffe *et al.*, 1993) and the other prevents

TABLE 3

Construction of Recombinant MVA: Comparison between BHK-21 and CEF Cells

Transfer vector[a]	Insertion site	Recombinant MVA foci[b]	
		CEF	BHK-21
pMC03	Deletion III	2×10^2	4×10^2
pLW22	Deletion II	ND	5×10^2
pMC1107Gus	*tk* locus	2×10^2	3×10^2

[a] pMC03 and pMC1107Gus both have GUS reporter genes (Carroll and Moss, 1995). pLW22 has a GAL reporter gene (L. Wyatt, unpublished).

[b] Recombinant MVA foci were identified by staining for GAL or GUS.

the association of actin tails with intracellular enveloped particles (Wolffe *et al.*, 1997). However, the correlation between MVA replication under multiple and single step growth conditions in the various cell lines, indicated that the primary block in semipermissive and nonpermissive cell lines was formation of infectious virus.

Electron microscopic studies confirmed the normal replication of MVA in BHK-21 cells and CEF. Both spherical immature and brick-shaped mature MVA particles were abundant in these permissive cell lines. In BS-C-1 cells, there were low numbers of mature particles consistent with the low yields of infectious virus. Some of the immature particles had small dense nucleoids thought to contain the viral DNA genome. Previous electron microscopic studies suggested that the principal defect in HeLa cells is the failure of immature MVA particles to form condensed cores and assume the characteristic brick shape (Sutter and Moss, 1992). In the present study, we found two types of immature MVA particles in HeLa cells: the majority were similar to those observed during a normal VV infection and appeared on cross-section as a circular membrane enclosing granular material and in some cases a nucleoid; others, however, were uniformly dense. The novel dense particles were frequently associated with cisternae and some were undergoing wrapping by cellular membranes. Wrapped dense particles were even found in extracellular spaces, suggesting that they had passed through the plasma membrane. It will be interesting to determine the biochemical and enzymatic composition of these particles and to evaluate whether they might be responsible for the spread of MVA to immediately adjacent HeLa cells as revealed by immunostaining. Some nonpermissive lines such as RAB-9, have an earlier block which prevented accumulation of significant numbers of immature enveloped virions. Reporter or viral gene expression studies suggested that the defect in RAB-9, PK(15), and RK13 cells involves inhibition of viral late protein synthesis. In the latter case, the defect was due to the absence of a functional K1L gene. The host restriction in CHO cells probably involves the absence of a functional CHO*hr* gene although this was not specifically examined.

Several aspects of this study pertain to the use of MVA as an expression vector. One reason for choosing MVA is the high degree of attenuation and inefficient propagation in human cells. A second reason is that MVA DNA replication and gene expression are relatively unimpaired, allowing use of strong late promoters for recombinant gene expression. However, the level of late gene expression varied and in some cells was less than with replication competent strains of VV. Nevertheless, in cells supporting the highest recombinant gene expression, the level achieved with the MVA and WR strains were similar and in others the difference was only several-fold. In some situations, MVA may have been chosen for recombinant gene expression because of expectations of lower CPE compared to standard VV strains. This is true for BS-C-1 and CV-1 cells. In some other cases, however, the CPE were delayed rather than prevented even in nonpermissive cells. Unfortunately, the cell lines that were most resistant to CPE, PK(15) and RAB-9, expressed viral or recombinant proteins poorly. If special cell lines are needed for specific studies, it would be prudent to first test them with recombinant MVA to determine the extent of CPE and reporter gene expression.

BHK-21 cells can be used for constructing and propagating recombinant MVA and therefore provides an alternative to primary CEF. A variety of transfer plasmids that facilitate expression cloning in the MVA genome have been described (Antoine *et al.*, 1996; Carroll and Moss, 1995; Scheiflinger *et al.*, 1996; Sutter and Moss, 1992; Wyatt *et al.*, 1996). These plasmids allow expression of one or two recombinant genes under moderate or strong promoters and some provide for color screening or antibiotic selection of recombinant viruses. Four regions of the MVA genome have been used for gene insertion: these consist of two sites in which spontaneous deletions had occurred during the adaptation of MVA to CEF, the hemagglutinin region, and the *tk* locus. Most of our own experience has been with recombination into the deletion sites. Nevertheless, as reported here, we had no difficulty isolating a tk^- recombinant MVA with either CEF or BHK-21 cells, suggesting that the many transfer plasmids previously constructed for use with standard strains of VV (Earl and Moss, 1991) may be suitable for MVA. However, we may have been fortunate in this one case since Scheiflinger *et al.* (1996) reported that tk^- MVA recombinants were difficult to purify in CEF, leading them to include a functional fowlpox virus *tk* gene in their transfer plasmids. Whether BHK-21 cells are superior to CEF for tk^- MVA remains to be determined.

Recent marker rescue experiments in our laboratory indicate that the MVA phenotype is the result of multiple gene defects that have an additive effect on host restriction (M. Carroll, C. Czerny, and B. Moss). This is an excellent result with regard to the safety of recombinant MVA, since spontaneous revertants are most unlikely. However, this result will surely complicate our efforts to understand the basis of MVA host restriction.

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REFERENCES

Ainsworth, S. K., and Karnovsky, M. J. (1972). An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* **20,** 225–229.

Altenburger, W., Süter, C.-P., and Altenburger, J. (1989). Partial deletion of the human host range gene in the attenuated vaccinia virus MVA. *Arch. Virol.* **105,** 15–27.

Antoine, G., Scheiflinger, F., Holzer, G., Langmann, T., Falkner, F. G., and Dorner, F. (1996). Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes. *Gene* **177,** 43–46.

Beattie, E., Kauffman, E. B., Martinez, H., Perkus, M. E., Jacobs, B. l., Paoletti, E., and Tartaglia, J. (1996). Host-range restriction of vaccinia virus E3L-specific deletion mutants. *Virus Genes* **12,** 89–94.

Bender, B. S., Rowe, C. A., Taylor, F., Wyatt, L. S., Moss, B., and Small, P. A. (1996). Oral immunization with a replication-deficient recombinant vacciinia virus protects mice against influenza. *J. Virol.* **70,** 6418–6424.

Bertholet, C., Drillien, R., and Wittek, R. (1985). One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. *Proc. Natl. Acad. Sci. USA* **82,** 2096–2100.

Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000 Dalton outer envelope protein. *J. Virol.* **65,** 5910–5920.

Carroll, M. W., and Moss, B. (1995). E. coli β-glucuronidase (GUS) as a marker for recombinant vaccinia viruses. *BioTechniques* **19,** 352–355.

Carroll, M. W., Overwiijk, W. W., Chamberlain, R. S., Rosenberg, S. A., Moss, B., and Restifo, N. P. (1997). Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: A murine tumor model. *Vaccine* **15,** 387–394.

Chang, H.-W., Uribe, L. H., and Jacobs, B. L. (1995). Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. *J. Virol.* **69,** 6605–6608.

Chang, H. W., Watson, J. C., and Jacobs, B. L. (1992). The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **89,** 4825–4829.

Dallo, S., Rodriguez, J. F., and Esteban, M. (1987). A 14K envelope protein of vaccinia virus with an important role in virus–host cell interactions is altered during virus persistence and determines the plaque size phenotype of the virus. *Virology* **159,** 423–432.

Drillien, R. D., Spehner, D., and Kirn, A. (1978). Host range restriction of vaccinia virus in Chinese hamster ovary cells: Relationship to shutoff of protein synthesis. *J. Virol.* **28,** 843–850.

Duncan, S. A., and Smith, G. L. (1992). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *J. Virol.* **66,** 1610–1621.

Earl, P. L., Cooper, N., and Moss, B. (1991). Preparation of cell cultures and vaccinia virus stocks. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl), pp. 16.16.1–16.16.7. Greene, Wiley Interscience, New York.

Earl, P. L., and Moss, B. (1991). Generation of recombinant vaccinia viruses. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl), pp. 16.17.1–16.17.16. Greene, Wiley Interscience, New York.

Engelstad, M., and Smith, G. L. (1993). The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* **194,** 627–637.

Gillard, S., Spehner, D., and Drillien, R. (1985). Mapping of a vaccinia virus host range sequence by insertion into the viral thymidine kinase gene. *J. Virol.* **53,** 316–318.

Hirsch, V. M., Fuerst, T. R., Sutter, G., Carroll, M. W., Yang, L. C., Goldstein, S., Piatak, M., Jr., Elkins, W. R., Alvord, W. G., Montefiori, D. C., Moss, B., and Lifson, J. D. (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: Effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* **70,** 3741–3752.

Hochstein-Mintzel, V., Huber, H. C., and Stickl, H. (1972). Virulenz und immunogenität eines modifizierten vaccinia-virus (Stamm MVA). *Z. Immun. Forsch.* **144,** 140–145.

Katz, S. L., and Broome, C. V. (1991). Vaccinia (smallpox) vaccine. Recommendations of the immunization practices advisory committee (ACIP). *Morbid. Mortal. Week. Rep.* **40,** 1–10.

Mahnel, H., and Mayr, A. (1994). Erfahrungen bei der schutzimpfung gegen orthopocken von mensch und tier mit dem impfstamm MVA. *Berl. Münch. Tierärzt. Wschr.* **107,** 253–256.

Martinez-Pomares, L., Stern, R. J., and Moyer, R. W. (1993). The ps/hr gene (B5R open reading frame homolog) of rabbitpox virus controls pock color, is a component of the extracellular enveloped virus, and is secreted into the medium. *J. Virol.* **67,** 5450–5462.

Mayr, A., Hochstein-Mintzel, V., and Stickl, H. (1975). Abstammung, eigenschaften und verwendung des attenuierten vaccinia-stammes MVA. *Infection* **3,** 6–14.

Mayr, A., Stickl, H., Müller, H. K., Danner, K., and Singer, H. (1978). Der pockenimpfstamm MVA: Marker, genetische struktur, erfahrungen mit der parenteralen schutzimpfung und verhalten im abwehrgeschwächten organismus. *Zbl. Bakt. Hyg.* **167,** 375–390.

Meyer, H., Sutter, G., and Mayr, A. (1991). Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* **72,** 1031–1038.

Moss, B., Rosenblum, E. N., Katz, E., and Grimley, P. M. (1969). Rifampicin: A specific inhibitor of vaccinia virus assembly. *Nature* **224,** 1280–1284.

Njayou, M., Drillien, R., and Kirn, A. (1982). Characteristics of the inhibition of protein synthesis by vaccinia virus in nonpermissive Chinese hamster ovary cells. *Ann. Virol.* **133E,** 393–402.

Oguiura, N., Spehner, D., and Drillien, R. (1993). Detection of a protein encoded by the vaccinia virus C7L open reading frame and study of its effect on virus multiplication in different cell lines. *J. Gen. Virol.* **74,** 1409–1413.

Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E. (1990). Vaccinia virus host range genes. *Virology* **179,** 276–286.

Ramsey-Ewing, A., and Moss, B. (1995). Restriction of vaccinia virus replication in CHO cells occurs at the stage of viral intermediate protein synthesis. *Virology* **206,** 984–993.

Ramsey-Ewing, A. L., and Moss, B. (1996). Complementation of a vaccinia virus host range K1L gene deletion by the non-homologous CP77 gene. *Virology* **222,** 75–86.

Rodriguez, J. F., and Smith, G. L. (1990). IPTG-dependent vaccinia virus: Identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res.* **18,** 5347–5351.

Scheiflinger, F., Falkner, F. G., and Dorner, F. (1996). Evaluation of the thymidine kinase (*tk*) locus as an insertion site in the highly attenuated vaccinia MVA strain. *Arch. Virol.* **141,** 663–669.

Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H. C., Schäfer, H., and Holzner, A. (1974). MVA-stufenimpfung gegen pocken. Kleinische erprobung des attenuierten pocken-lebendimpfstoffes, stamm MVA. *Dtsch. Med. Wschr.* **99,** 2386–2392.

Sutter, G., and Moss, B. (1992). Nonreplicating vaccinia vector efficiently

expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* **89**, 10847–10851.

Sutter, G., Ohlmann, M., and Erfle, V. (1995). Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Lett.* **371**, 9–12.

Sutter, G., Wyatt, L. S., Foley, P. L., Bennink, J. R., and Moss, B. (1994). A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* **12**, 1032–1040.

Wolffe, E. J., Isaacs, S. N., and Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* **67**, 4732–4741.

Wolffe, E. J., Katz, E., Weisberg, A., and Moss, B. (1997). The A34R glycoprotein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. *J. Virol.* **71**, 3904–3915.

Wolffe, E. J., Moore, D. M., Peters, P. J., and Moss, B. (1996). Vaccinia virus A17L open reading frame encodes an essential component of nascent viral membranes that is required to initiate morphogenesis. *J. Virol.* **70**, 2797–2808.

Wyatt, L. S., Moss, B., and Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**, 202–205.

Wyatt, L. S., Shors, S. T., Murphy, B. R., and Moss, B. (1996). Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* **14**, 1451–1458.

61. Inlow, D., Shauger, A., and Maiorella, B. (1989). *J. Tissue Culture Meth.*, **12**, 13–16.
62. Tramper, J., van den End, E. J., de Gooijer, C. D., Kompler, R., van Lier, F. L. J., Usmany, M., and Vlak, J. M. (1990). *Ann. NY Acad. Sci.*, **589**, 423.
63. Weiss, S. A., Gorfien, S., Fike, R., DiSorbio, D., and Jayme, P. (1990). *Proceedings of 9th Australian Biotechnology Conference*, p. 230. University of Queensland Press.
64. Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Sacki, Y., Sato, Y., and Furusawa, M. (1985). *Nature*, **315**, 592.
65. Overton, H. A., Ihara, T., and Bishop, D. H. L. (1987). *Virology*, **157**, 338.
66. Takehara, K., Ireland, D., and Bishop, D. H. L. (1988). *J. Gen. Virol.*, **69**, 2763.
67. Jarvis, D. L., Fleming, J., Kovacs, G. R., Summers, M. D., and Guarino, L. (1990). *Bio/Technology*, **8**, 950.
68. Patel, G., Nasmyth, K., and Jones, N. (1992). *Nucl. Acids Res.*, **20**, 97–104.
69. Peakman, T. C., Harris, R. A., and Gewert, D. R. (1992). *Nucl. Acids Res.*, **20**, 495–500.

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EXHIBIT A

9

Expression of genes by vaccinia virus vectors

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1. Introduction

Jenner introduced the use of cowpox virus as a vaccine against smallpox in 1798, and, as he predicted, this practice led to the eradication of smallpox (in 1977), although in the modern era it was vaccinia, not cowpox, virus which was used as the smallpox vaccine. Today, variola (smallpox) virus is confined to two high-security laboratories in Moscow and Atlanta, and the World Health Organization plans to destroy these last stocks in 1993 after the genomes of representative strains have been sequenced. Cowpox and vaccinia viruses have clearly played important roles in the history of human medicine, since they represent the first human vaccine and the only vaccine whose use has resulted in the eradication of a human disease. Surprisingly, interest in poxviruses in general, and vaccinia virus in particular, increased substantially after the disappearance of smallpox. This interest stemmed, in a large part, from the development of vaccinia virus as an expression vector and from the potential use of vaccinia virus recombinants as live vaccines against pathogens other than variola virus. In this chapter, concepts and protocols central to the use of vaccinia virus as an expression vector are described. Readers should refer to another chapter in this series (1), which provides an earlier account of this topic and contains several protocols which remain essentially unaltered, and to another recent review on the construction and applications of vaccinia virus recombinants (2).

2. Vaccinia virus biology

Excellent reviews have been written recently on poxvirus molecular biology (3) and pathogenesis (4) and on the enzymology of vaccinia virus transcription (5) and DNA replication (6), and these should be referred to by interested readers. Only brief outlines of these processes are included in this chapter, with emphasis on those concepts necessary for the use of vaccinia virus as an expression vector.

2.1 Virus structure

Poxviruses are the largest animal viruses and their complexity is illustrated by the presence of more than 100 proteins within the virus particle. Vaccinia virus is the prototype of the *Orthopoxvirus* genus, and is approximately 250 by 350 nm in size. The biconcave core contains the DNA genome and many virus-coded enzymes, and is flanked by lateral bodies. Two forms of infectious virus exist. One form, termed intracellular naked virus (INV), is found in the cytoplasm of virus-infected cells and represents the great majority of infectious progeny: 99% of the total progeny of the WR strain of vaccinia virus is cell-associated. The second form, termed extracellular enveloped virus (EEV), is released from infected cells and possesses an additional lipid envelope which is acquired from Golgi membrane. Several virus-coded glycoproteins are present only in this envelope (7) and give EEV its distinct immunological and biological properties. In particular, EEV production is required for plaque formation and for the efficient spread of virus *in vitro* and *in vivo* (7).

2.2 Virus genome

The vaccinia virus genome is a large double-stranded DNA molecule of 180–200 kbp, the precise size depending on virus strain. It has a base composition of 67% A+T and is non-infectious when de-proteinized. The termini of the two DNA strands are covalently joined into one polynucleotide chain by a single-stranded DNA hairpin. The terminal regions exhibit considerable heterogeneity that may result from deletions, duplications, or transpositions. In contrast, the central two-thirds of the genome show high conservation among different orthopoxviruses and contain the genes essential for virus replication. The complete 192 kbp sequence of the Copenhagen strain of vaccinia virus (8) and much of that of the laboratory WR strain have been reported. The genome contains about 200 genes whose protein-coding sequences are packed tightly, displaying only short intergenic regions or, in some cases, limited overlaps. Deletions of specific genes or of larger regions of DNA near the termini (9) have shown that approximately one-third of the gene complement is non-essential for virus replication *in vitro*.

2.3 Enzymes

Poxviruses have evolved to replicate in the cytoplasm and consequently encode a multitude of replicative and transcriptional enzymes which permit replication to proceed independently of the host cell nucleus. Many of these enzymes are packed within the virus particle and are essential for initiation of infection, while others are present only within the infected cell. The most important virion enzyme is the multi-subunit DNA-dependent RNA polymerase which recognizes promoters only from poxviruses. Thus, the protein-

coding regions of foreign genes are linked to vaccinia virus promoters to enable their expression. For DNA replication, the virus encodes a DNA polymerase, a topoisomerase, and a DNA ligase, in addition to several enzymes involved in the synthesis of nucleoside triphosphate precursors. Other exotic enzymes, such as the steroid hormone biosynthetic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), are also encoded by vaccinia virus.

2.4 Gene expression

The virus genes are expressed in strict temporal fashion. Immediately after infection, the virus-associated DNA-dependent RNA polymerase transcribes only the early class of genes. Some of these genes encode enzymes needed for DNA synthesis, and others encode virulence factors or proteins that mediate further uncoating of the particle to permit DNA replication. At the onset of DNA replication, transcription of most early genes stops and an intermediate class of genes is activated. Intermediate transcription requires prior synthesis of early proteins, but is not strictly dependent upon DNA replication, since it may occur from plasmid templates that are transfected into virus-infected cells in which DNA replication is blocked. At least three intermediate proteins are transcription factors which are required for late transcription (10). Late genes are distinct from intermediate genes in that they are absolutely dependent upon prior DNA synthesis. They encode late structural proteins which constitute the new virus particles, and also some enzymes and early transcription factors which are packaged into maturing particles. A fourth class of gene is termed constitutive because it is expressed throughout infection owing to the presence of early and late promoters upstream from the open reading frame (ORF).

2.5 Morphogenesis

Poxvirus replication occurs in distinct cytoplasmic factories. Various stages of morphogenesis may be seen in the electron microscope and these are shown in *Figure 1*. The first structures formed are lipid crescents which contain virus protein. These enlarge into complete ovals containing the condensing nucleoprotein core, and then mature into electron-dense infectious INV particles. A small fraction of INV becomes enveloped by a double layer of Golgi-derived membrane, and the particle migrates to the cell surface, where the outer layer fuses with the plasma membrane and releases EEV from the cell.

3. Construction of vaccinia virus recombinants

The large and non-infectious nature of the vaccinia virus genome has made it impracticable to ligate foreign DNA directly into the genome and recover infectious virus. Moreover, foreign protein-coding sequences need to be positioned downstream from a vaccinia virus promoter to ensure efficient

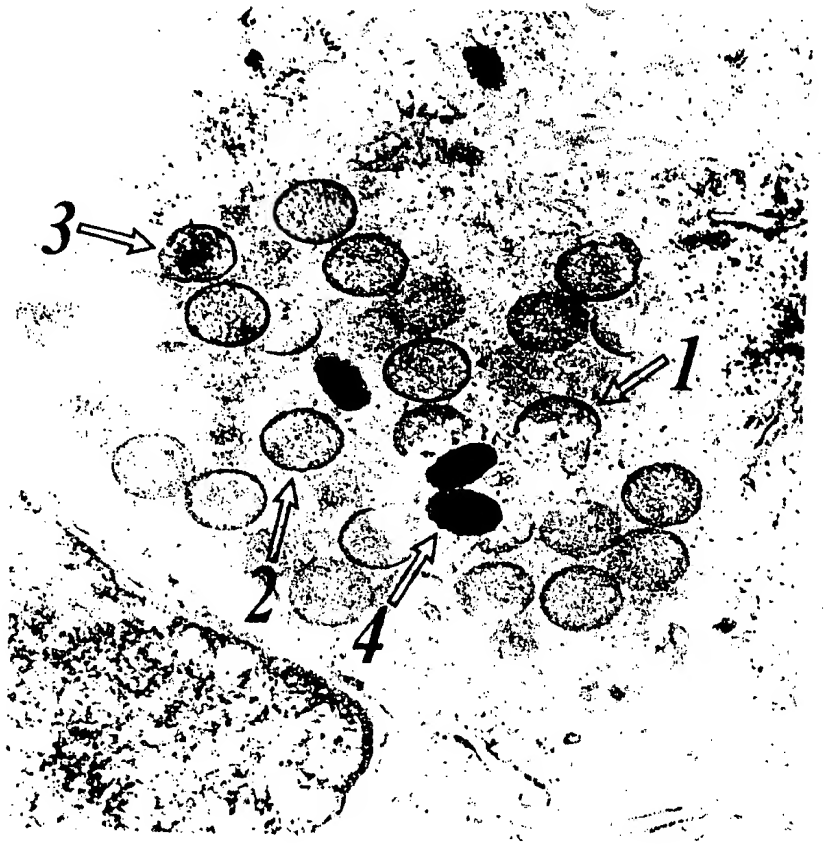

Figure 1. Electron micrograph of a vaccinia virus cytoplasmic factory. Numbers 1–4 show stages of morphogenesis starting from lipid crescent (1) to INV (4). (Reproduced from ref. 29 with permission from Oxford University Press.)

expression. In view of these practical difficulties, recombinant vaccinia viruses are constructed by the two-step procedure illustrated in *Figure 2*. A plasmid vector is constructed in which the foreign gene is linked to a vaccinia virus promoter, and then the gene is transferred into the virus genome by homologous recombination in virus-infected cells transfected with the recombinant plasmid.

3.1 Plasmid vectors

Numerous plasmid insertion vectors have been described for transfer of foreign genes into vaccinia virus. The choice of vector determines the onset, level, and duration of gene expression, the site in the genome at which the foreign gene is inserted, and the method by which recombinants are selected. Careful consideration must be given to these parameters. The simplest

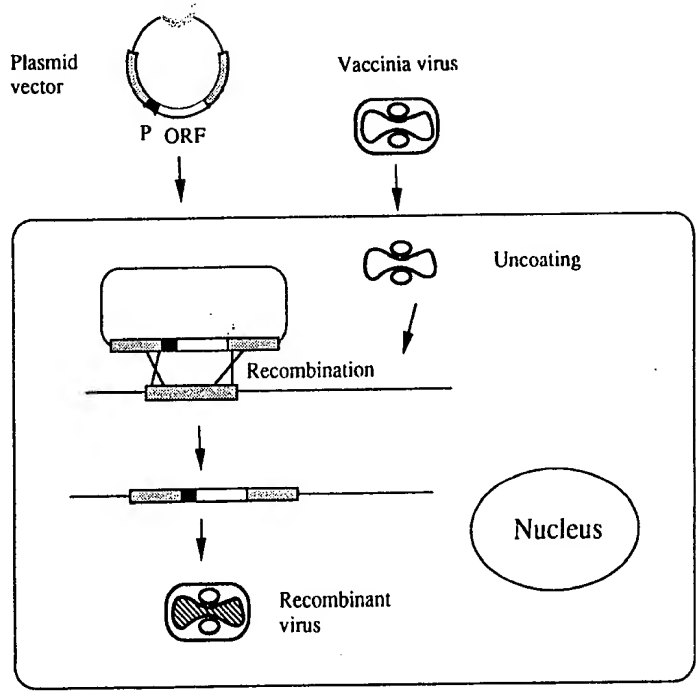

Figure 2. Schematic representation of the formation of recombinants. A plasmid vector containing the ORF to be expressed linked to a vaccinia virus promoter (P) is transfected into cells infected with vaccinia virus. Recombination between the vaccinia virus DNA flanking the gene and the corresponding DNA from the virus genome inserts the foreign gene into the virus genome. This is replicated and packaged into infectious progeny virus.

plasmid vectors have structures illustrated by vector pRK19 (11) in *Figure 3*. A vaccinia virus promoter, in this case from the vaccinia virus late gene encoding the structural protein p4b, is positioned immediately upstream from several unique restriction endonuclease sites into which a foreign gene can be inserted. The promoter and cloning sites are flanked by DNA from a non-essential locus in the virus genome, in this case the thymidine kinase gene (*tk*), which directs insertion of the foreign gene to this site. The *tk* locus has been used extensively, since interruption of this gene facilitates selection of recombinants (see Section 3.4.1).

Vector pRK19 and many other vectors, such as pGS20 (12), pSC11 (13), and pMJ601 (14), do not have an ATG codon positioned downstream from the vaccinia virus promoter to which foreign sequences can be fused, and are therefore suitable for expression of complete ORFs which provide their own translational control signals. Other vectors have an ATG codon positioned upstream from restriction sites which are arranged in all three reading frames and are followed by translational termination codons in all reading frames to enable straightforward expression of any protein-coding sequence (15, 16).

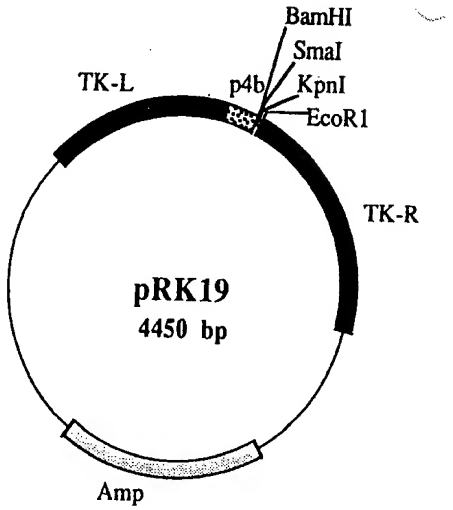

Figure 3. Structure of pRK19 (11), a typical plasmid vector for construction of a recombinant expressing a foreign gene. The p4b vaccinia virus promoter is positioned upstream of restriction endonuclease sites, and the region is flanked by DNA from the left (*tk*-L) and right (*tk*-R) of *tk*.

3.2 Choice of promoters

The promoter determines the level of foreign gene expression and the time during infection at which the gene is expressed. The strongest natural vaccinia virus promoters are from genes encoding major late proteins that form the new virus particles, such as the 11 kDa (17) and p4b genes (18). Vectors enabling even higher levels of expression are described in Section 5. A constitutively expressed promoter from a gene within the inverted terminal repetition of vaccinia virus strain WR which encodes a polypeptide of 7.5 kDa has been used widely (12). (For a list of other poxvirus promoters that have been used see ref. 2.) In constructing a transfer vector, the promoter of the foreign gene should be removed so that the protein-coding sequence can be placed as close as possible to the transcriptional start site of the vaccinia virus promoter. Additional ATG codons upstream from the ORF should also be removed.

In some situations it is important to express the foreign gene either early or late during infection. If the foreign gene contains the early transcriptional termination sequence TTTTTNT within its coding region, the majority of early transcripts will terminate approximately 50 nucleotides downstream from this site and full-length protein may not be produced. In such a situation it is desirable either to express the gene from a late promoter so that premature termination does not occur (19) or to remove termination sequences by mutagenesis (20). Conversely, there are some situations in which early

expression is important. For instance, late during vaccinia virus infection the presentation of some peptide epitopes to class I restricted cytotoxic T lymphocytes (CTLs) is blocked, whereas the same epitopes may be presented early during infection (21, 22). If recombinants are constructed in order to determine whether the expressed antigen is recognized on the surface of infected cells by CTLs, it is sensible, and may be essential, to express the antigen from an early promoter.

3.3 Insertion sites

The vaccinia virus genome contains numerous sites that are non-essential for virus replication *in vitro*, into which foreign genes may be inserted. These are listed in *Table 1*. While the endogenous genes present at these sites are non-essential *in vitro*, in many cases the encoded protein plays an important role in virulence of the virus *in vivo*. Virus attenuation resulting from deletion of a specific gene, or group of genes, may diminish the immune response following virus infection, and this may be important if the recombinant is used to raise an antibody or T-cell response against the foreign antigen or is tested as a candidate vaccine. For most laboratory applications where the recombinant is to be used to infect cells *in vitro*, however, the site of insertion is less important than the kinetics and level of gene expression.

The most convenient and widely used site is *tk*, since insertion here allows tk^- recombinants to be selected on tk^- cells in the presence of 5-bromodeoxyuridine (BUdR) (23). Several other genes involved in nucleic acid metabolism, such as those encoding thymidylate kinase, ribonucleotide reductase, and DNA ligase, are also non-essential for virus replication, but, like *tk* (24), they affect virus virulence *in vivo*. Other non-essential virulence factors include the vaccinia virus growth factor (25), a 13.8 kDa secretory virokine (26), a secretory protein which interferes with complement function (27), and the biosynthetic steroid enzyme 3β-HSD (28). Since both the INV and EEV forms of virus particle are infectious, the genes required for the formation and release of EEV are also non-essential for production of infectious virus. Some, however, are required for plaque formation: the 14 kDa fusion protein, a 37 kDa acylated protein, and a lectin-like glycoprotein (29–31). Many other individual genes and blocks of genes adjacent to the genomic termini (9) are non-essential for virus replication *in vitro*. If the insertion site is not important for other reasons, however, foreign genes are better inserted into the central region of the genome, since it is more stable than the variable termini.

3.4 Methods for selecting recombinants

Recombinants are formed by homologous recombination within cells infected with vaccinia virus and transfected with a plasmid vector, as described in *Protocol 1*. This method produces recombinants at a frequency of approximately

Table 1. Sites for inserting foreign DNA into the vaccinia virus genome

Gene name[a]	Function	Reference
C23L-K1L		(9, 16)
C13L[b]	7.5 kDa protein	(57)
C11R[g]	Vaccinia virus growth factor[c]	(25)
C3L[g]	Complement control protein	(27)
N1L[g]	13.8 kDa secretory virokine	(26)
M1L		(58)
M2L		(58)
K1L	Human host range protein	(58)
K2L	Serine protease inhibitor	(58, 59)
K3L	eIF-2α homologue, interferon resistance	(60)
K4L		(30, 58)
K7R		(58)
F1L		(58)
F4L	Ribonucleotide reductase (small subunit)	(58)
F9L		(58)
F13L	EEV envelope antigen	(30)
E1L		(58)
I4L[g]	Ribonucleotide reductase (large subunit)	(61)
J2R[g]	Thymidine kinase[d]	(23)
D8L	35 kDa membrane protein	(62)
A26L/A27L[e]	A-type inclusion body protein	(50)
A27L	14 kDa fusion protein, needed for virus egress	(29)
A34R (*Sal*L4R)	Lectin-like EEV envelope glycoprotein	(31)
A40R (*Sal*L2R)	Lectin-like glycoprotein	(Duncan and Smith, unpublished data)
A42R	Profilin	(47)
A43 (*Sal*F5R)	Glycoprotein	(63)
A44L (*Sal*F7L)[g]	3-β-hydroxy steroid dehydrogenase	(28)
A48R (*Sal*F11R)[g]	Thymidylate kinase	(64)
A50R (*Sal*F13R)[g]	DNA ligase	(42, 65)
A56R	Haemagglutinin	(66, 67)
B5R	Complement control protein	
	Host range	(68)
B12R	Protein kinase-related	(Banham and Smith, unpublished data)
B13R[e]	Serine protease inhibitor	(69)
B22R[f]	Serine protease inhibitor	(69)
B15R-B29R		(9)

[a] Gene names relate to strains Copenhagen (8) or WR (shown in brackets) (70). The nomenclature indicates the *Hin*dIII restriction fragment containing the gene (fragments are labelled alphabetically in order of decreasing size) and the direction of transcription (R or L). *Sal*I nomenclature is used for the right side of the WR *Hin*dIII A fragment because the sequence to the left is incomplete.
[b] In some virus strains (for example, WR) this gene is located within the inverted terminal repeat.
[c] In some virus strains this is a 35 kDa secreted protein and maps within the inverted terminal repeat.
[d] The majority of recombinants have been generated by insertion into this gene.
[e] In the Copenhagen strain this gene is interrupted.
[f] In the Copenhagen strain this gene is present at the left end of the genome and is named C12L.
[g] The gene product is known to increase virus virulence.

0.1%; selective methods are, therefore, needed to distinguish recombinant from parental virus. Many different approaches have been used, and only those in widespread use are considered here; for a comprehensive list the reader should see ref. 2.

Protocol 1. Transfection procedure for producing recombinants

Materials

- CV-1 cells: obtainable from the American Type Culture Collection (ATCC) as CCL-70
- MEM: minimal essential medium (Gibco) containing 100 U/ml penicillin and 100 μg/ml streptomycin
- wild type (WT) vaccinia virus: for example the WR strain obtainable from the ATCC as VR-119
- plasmid DNA containing the foreign gene cloned downstream from a vaccinia virus promoter
- carrier DNA: 1 mg/ml sonicated salmon sperm or calf thymus DNA

Method

1. Grow CV-1 cell monolayers in MEM containing 8% (v/v) fetal calf serum (FCS) in a 25 cm^2 flask to approximately 80% confluence.
2. Aspirate the medium and infect the cells with WT vaccinia virus at a multiplicity of infection (m.o.i.) of 0.05 plaque-forming units (p.f.u.)/cell. Gently rock the flask at 37°C for 2 h.
3. Precipitate 1 μg of plasmid DNA and 19 μg of carrier DNA in a total volume of 1 ml with calcium phosphate (see Chapter 8, *Protocol 6*).
4. Remove the virus inoculum and add the calcium phosphate-precipitated DNA, then incubate the flask for 30 min at 37°C.
5. Add 9 ml of MEM containing 8% FCS and incubate the flask for 3–4 h at 37°C.
6. Replace the medium with fresh MEM containing 8% FCS.
7. Scrape the cells into the medium at 48 h post infection (p.i.), pellet them at 2000 *g* for 5 min and resuspend the cell pellet in 1 ml of MEM containing 8% FCS. Aliquot and store the cells at −70°C.

3.4.1 Selection of *tk*⁻ virus

The most widely used method of generating recombinants is to insert the foreign DNA into the *tk* locus and select *tk*⁻ recombinants, as described

in *Protocol 2* (12, 23). Despite its popularity, this method has some limitations:

- it requires a tk^+ parental virus
- it allows insertion only into *tk*
- recombinant plaques must be selected on tk^- cells in the presence of the mutagenic compound BUdR
- some tk^- plaques are spontaneous mutants which do not contain the foreign gene

Spontaneous tk^- mutants may be distinguished from recombinants by a variety of means, including DNA hybridization (*Protocol 3*), immunological screening for expression of the foreign antigen, or polymerase chain reaction (PCR; *Protocol 4*). The latter approach is fast and uses oligonucleotide primers that bind to either side of the insertion site within *tk*.

Protocol 2. Selection of tk^- virus

Materials

- an aliquot of infected cells produced in *Protocol 1*
- 5 mg/ml BUdR; filter-sterilize, aliquot, and store at −20°C
- 2% (w/v) low gelling temperature agarose: autoclave
- human tk^-143 cells
- 2 × MEM (Gibco)
- 1% (w/v) neutral red stain
- 20 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in dimethylformamide

Method

1. Thaw the aliquot of infected cells and freeze–thaw twice more. Sonicate briefly in a sonic bath to complete cell lysis and disaggregate the virus.
2. Prepare ten-fold dilutions of virus to a final dilution of 10^{-4} in MEM containing 8% FCS.
3. Remove the medium from confluent monolayers of tk^-143 cells in 50 mm Petri dishes and add 0.5 ml of diluted virus. Plate out the 10^{-2}, 10^{-3}, and 10^{-4} dilutions in duplicate. Gently rock the dishes for 2 h at 37°C.
4. Remove the virus inocula and overlay the cells with 4 ml of MEM containing 1% low gelling temperature agarose, 2.5% FCS, and 25 μg/ml BUdR. Leave the dishes at room temperature in the dark until the agarose has set and then incubate them for 2 days at 37°C.

5. Stain the virus plaques by overlaying the dishes with 4 ml of MEM containing 0.01% neutral red and 1% low gelling temperature agarose, and incubate for 2–4 h at 37°C. (If the recombinant is derived from vector pSC11 or other plasmids which insert *Escherichia coli lacZ* into *tk*, include 200 μg/ml X-gal in the overlay. This compound is converted by β-galactosidase into a deep blue colour, allowing recombinants to be distinguished from spontaneous tk^- mutants.) Use Pasteur pipettes to pick plaques from the highest dilution at which they are visible. Store the plaques at −70°C in a small volume of MEM containing 8% FCS.

Protocol 3. Screening tk^- plaques by DNA hybridization

Materials

- tk^- plaques isolated in *Protocol 2*
- phosphate-buffered saline (PBS): 170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)
- nitrocellulose membrane (Schleicher and Schuell)
- 20 × SSC: 3 M NaCl, 0.3 M trisodium citrate
- 50 × Denhardt's solution: 1% (w/v) bovine serum albumin (fraction V), 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone
- 10% (w/v) sodium dodecyl sulphate (SDS)
- a ^{32}P-labelled DNA probe specific for the foreign gene

Method

1. Use half of the yield from tk^- plaques to infect tk^-143 monolayers in 24-well plates. Incubate the plates for 2 h at 37°C. Add 1 ml of MEM containing 2.5% FCS and 25 μg/ml BUdR. Incubate the plates for 2 days at 37°C.
2. Check the cells by microscopy for cytopathic effect (c.p.e.). If more than 50% of the cells show c.p.e., scrape the cells into the medium using the plunger from a 1 ml syringe.
3. Transfer the cells to a 1.5 ml tube and centrifuge at 12 000 *g* for 1 min.
4. Discard the supernatant and resuspend the cells in 200 μl of PBS. Freeze–thaw three times, sonicate briefly, and then load 50 μl on to a nitrocellulose membrane. Place the membrane for 3 min on a filter paper soaked with 0.5 M NaOH, transfer for 3 min to a second filter paper soaked with 1 M Tris–HCl (pH 7.5), and then for 3 min to a third filter paper soaked with 2 × SSC. Air-dry the membrane and bake it for 2 h at 80°C in a vacuum oven.

Protocol 3. *Continued*

5. Hybridize the membrane with the radioactive probe.
   (a) Incubate the filter in 6 × SSC containing 5 × Denhardt's solution for 4 h at 65°C.
   (b) Denature the probe by boiling for 2 min, add it to the solution and incubate for 12–18 at 65°C.
   (c) Wash the membrane twice for 15 min each in 2 × SSC, 0.1% SDS at 65°C, and twice for 15 min each in 0.2 × SSC at 65°C.
   (d) Air-dry the membrane and expose it to X-ray film.
6. Select positive isolates by reference to the developed film.
7. Plaque-purify the recombinants further. A total of three cycles of plaque purification usually gives homogeneous isolates. Prepare and titrate virus stocks. Store them at −70°C.

Protocol 4. Screening tk^- plaques by PCR

Materials

- tk^- plaques isolated in *Protocol 2*
- 20 μM solutions of oligonucleotide primers 1 and 2 (flanking either side of the insertion site within *tk*)
- 5 U/ml *Taq* polymerase
- 10 × PCR buffer: 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin
- dNTP solution: 2 mM each of dATP, dCTP, dGTP, and dTTP

Method

1. Carry out steps 1–3 of *Protocol 3*. Resuspend the infected cells in 400 μl of water. Freeze–thaw the cells twice and sonicate them.
2. Heat the disrupted cells at 95°C for 5 min and then place them on ice.
3. Set up the PCR in a final volume of 50 μl by mixing the following:

• infected cell extract	3 μl
• oligonucleotide primer 1	2.5 μl or 5 μl of 10 μM
• oligonucleotide primer 2	2.5 μl or 5 μl of 10 μM.
• dNTP solution	5 μl
• 10 × PCR buffer	5 μl
• *Taq* polymerase	0.25 μl
• water	32 μl

4. Incubate the mixture at 92°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. Repeat this cycle 24 times. The hybridization temperature depends upon the primers used.
5. Electrophorese 10–20 μl of the product on a 1% (w/v) agarose gel including size markers. The size of the PCR product indicates whether the plaque is a spontaneous tk^- mutant or a recombinant with exogenous DNA inserted into *tk*.
6. Carry out step 7 of *Protocol 3*.

3.4.2 Expression of β-galactosidase

To expedite the distinction between tk^- recombinants and spontaneous tk^- mutants selected in the presence of BUdR, some vectors contain *lacZ* linked to a vaccinia virus promoter which may be co-inserted into *tk* (13). In the presence of the chromogenic substrate X-gal, plaques formed by recombinants can be distinguished visually from spontaneous mutants by their blue colour (see *Protocol 2*). Indeed, expression of β-galactosidase can be used alone to detect recombinants without the need for other selection methods (32). Moreover, subsequent replacement of *lacZ* by another foreign gene enables the selection of recombinants which no longer form blue plaques (32).

3.4.3 Selection of tk^+ virus

An alternative method for selecting recombinants is to insert herpes simplex virus type 1 (HSV-1) *tk* linked to a vaccinia virus promoter into any non-essential site of a tk^- vaccinia virus genome (23). Recombinants are tk^+ and will form plaques in tk^- cells in the presence of aminopterin (methotrexate), as described in *Protocol 5*. A second foreign gene may be inserted simultaneously with HSV-1 *tk* and selected in the same way (33). Unlike the selection of tk^- virus using BUdR, this system produces only true recombinants and does not require the use of mutagenic compounds. It remains dependent, however, upon use of a tk^- cell line.

Protocol 5. Selection of tk^+ virus

Materials

- 1 mM aminopterin (methotrexate)
- 40 × TAGG: 0.6 mM thymidine, 2 mM adenosine, 2 mM guanosine, 0.4 mM glycine
- filter-sterilize, aliquot, and store these solutions at −20°C
- a tk^- vaccinia virus

Protocol 5. *Continued*

Method

1. Complete *Protocol 1* using the tk^- virus and an appropriate plasmid (such as pVPHTK2: see ref. 23) in steps 1–3 of *Protocol 2*.
2. Remove the virus inoculum and overlay the cells with MEM containing 1% low gelling temperature agarose, 2.5% FCS, 0.1 mM non-essential amino acids, 1 × TAGG and 1 μM aminopterin. Leave the plates at room temperature in the dark until the agarose has set and then incubate them for 2 days at 37°C.
3. Carry out step 5 of *Protocol 2*.
4. Carry out step 7 of *Protocol 3*.

3.4.4 Neomycin resistance

Another dominant marker gene used to select recombinants is the neomycin resistance gene. The antibiotic G418 inhibits production of WT virus but allows plaque formation by virus expressing this gene (34). This selection system has been used infrequently (16).

3.4.5 Expression of the *E. coli* guanine phosphoribosyltransferase gene (*Ecogpt*)

This selectable marker gene is now widely used to make recombinants and for experimental manipulation of the vaccinia virus genome. Mycophenolic acid (MPA) inhibits inosine monophosphate dehydrogenase and results in blockage of purine synthesis and inhibition of vaccinia virus replication in most cell lines. The blockage may be overcome by expressing *Ecogpt* from a constitutive vaccinia virus promoter and providing the substrates xanthine and hypoxanthine (15, 35), as described in *Protocol 6*. Some workers have found it necessary to include aminopterin, to block *de novo* synthesis of purines, and thymidine (35). The system is versatile and overcomes the deficiencies of selecting tk^- viruses with BUdR. It is not restricted to insertion into a particular site in the virus genome, does not require the use of specific cell lines or mutagenic compounds, and allows plaque formation by recombinants but not by spontaneous mutants.

An additional advantage of *Ecogpt* is that it is possible to select against its expression, as described in *Protocol 7*, so that $Ecogpt^-$ recombinants may be derived from an $Ecogpt^+$ parent (36). Reverse selection requires the use of a hypoxanthine phosphoribosyltransferase negative ($hprt^-$) cell line, since the nucleoside analogue 6-thioguanine is toxic for mammalian cells expressing *hprt*. Combined with positive selection for *Ecogpt*, reverse *Ecogpt* selection enables a mutation to be introduced into a specific site without leaving the selectable marker in the vaccinia virus genome. Consequently, multiple mutations may be built sequentially into the same recombinant.

Protocol 6. Selection of recombinants expressing *Ecogpt*

Materials

- CV-1, human tk^-143 or BSC-1 cells: BSC-1 cells are available from the ATCC as CCL-26
- 10 mg/ml MPA: filter-sterilize, aliquot, and store at −20°C (0·1M NaOH)
- 10 mg/ml xanthine in 0.1 M NaOH: filter-sterilize, aliquot, and store at −20°C
- 10 mg/ml hypoxanthine: filter-sterilize, aliquot, and store at −20°C

0·1M NaOH

Method

1. Construct a plasmid containing *Ecogpt* linked to a vaccinia virus promoter (derived, for instance, from pGpt07/14: see ref. 35) inserted into the vaccinia virus gene of interest. Carry out *Protocol 1* to obtain recombinants containing *Ecogpt*.
2. Prepare monolayers of CV-1, BSC-1, or tk^-143 cells in 50 mm Petri dishes. Replace the growth medium with MEM containing 2.5% FCS, 25 μg/ml MPA, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine. Incubate the dishes for 12–24 h at 37°C.
3. Remove the medium and infect the cells with appropriate dilutions of virus (10^{-2}, 10^{-3}, and 10^{-4} dilutions if the virus is obtained directly from *Protocol 1*). Gently rock the dishes for 2 h at 37°C.
4. Remove the virus inocula and overlay the cells with MEM containing 1% low gelling temperature agarose, 2.5% FCS, 25 μg/ml MPA, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine. Leave the dishes at room temperature in the dark until the agarose has set and then incubate them for 2–3 days at 37°C.
5. Carry out step 5 of *Protocol 2*.
6. Carry out step 7 of *Protocol 3*.

This method is taken from ref. 15. Other investigators (35) have reported that the selection system does not work on CV-1 cells in the absence of aminopterin (0.2 μg/ml) and thymidine (4 μg/ml). works with BSC1 + Vero without aminopterin.

Protocol 7. Selection against *Ecogpt* expression

Materials

- 1 mg/ml 6-thioguanine: filter-sterilize, aliquot, and store at −20°C
- an $hprt^-$ cell line such as HeLa D98R or murine STO: available from the Sir William Dunn School of Pathology or the ATCC (CRL 1503), respectively

Protocol 7. *Continued*

Method

1. Construct a plasmid containing sequence for replacing *Ecogpt* present in a recombinant obtained in *Protocol 6*. Carry out *Protocol 1* to obtain recombinants lacking *Ecogpt*.
2. Grow monolayers of *hprt*$^-$ cells in 50 mm Petri dishes. Remove the growth medium and infect the cells with the virus yield from step 1, using a range of virus dilutions to obtain an appropriate number of *Ecogpt*$^-$ plaques. Gently rock the dishes for 2 h at 37°C.
3. Remove the virus inocula and overlay the cells with MEM containing 1% low gelling temperature agarose, 2.5% FCS, and 1 μg/ml 6-thioguanine. Allow the agarose to set at room temperature and incubate them for 3 days at 37°C. Plaques formed on HeLa D98R cells are smaller than on those formed on BSC-1 cells, and it is therefore necessary to incubate infected D98R cells for a minimum of 3 days.
4. Carry out step 5 of *Protocol 2*.
5. Carry out step 7 of *Protocol 3*.

3.4.6 Use of conditional lethal mutant viruses

Identification of vaccinia virus genes that are required for growth on certain cell types has provided another dominant system for the selection of recombinants. The parental virus, which lacks the host range gene and must be grown on an alternative permissive cell type, is recombined with a plasmid that contains the host range gene and a second gene for co-insertion. Recombinants are selected on the cell line non-permissive for the parental virus. Vectors have been described which use a vaccinia virus human host range gene for selection and which allow expression of foreign protein-coding sequences linked to another vaccinia virus promoter (16).

Other conditional lethal viruses, such as temperature-sensitive or drug-dependent mutants, have been used to select for recombinants (37, 38). If cells are infected with such a virus under non-permissive conditions and then transfected with WT virus DNA together with a recombinant plasmid, viable virus is produced only in those cells which take up both WT virus DNA and the recombinant plasmid. The WT genome is transcribed by *trans*-acting functions provided by abortive infection with the conditional lethal mutant and recombines very efficiently with the co-transfected plasmid, so that up to 30% of the progeny virus is recombinant (38).

3.4.7 Transient dominant selection

The mechanism by which recombinants are formed from parental virus and transfected plasmid was originally thought to occur by double homologous recombination on either side of the insertion site. In this way, the WT allele

present in the virus genome is simply replaced by the mutant allele from the plasmid. It has become clear, however, that recombinant genomes can also be formed by a single homologous recombination event on one side of the foreign gene, so that the entire plasmid is inserted into the virus genome (39, 40). As shown in *Figure 4*, such virus genomes contain direct repeats of vaccinia virus DNA and are consequently unstable. They may resolve by recombination between the direct repeats to remove one copy of the repeat and the intervening DNA, yielding either parental or recombinant virus.

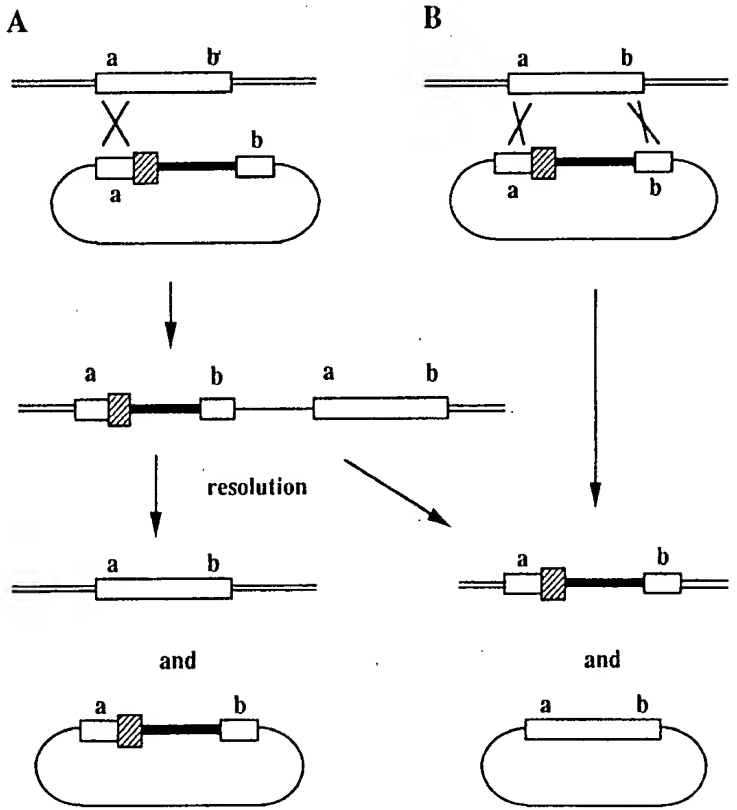

Figure 4. Formation of recombinants by single (A) or double (B) homologous recombination events. In (A) a single recombination event is shown to the left of the foreign gene between homologous virus sequences designated 'a'. This produces an intermediate virus that contains direct repeats of sequences 'a' and 'b'. Owing to the instability of the virus that contains direct repeats of sequences 'a' and 'b'. Owing to the instability of the direct repeats, the virus undergoes recombination between the 'a' or 'b' repeats. In the former case, the products of the resolution event are the WT virus genome and the plasmid that was used for transfection. In the latter case the products are a recombinant genome and a plasmid containing the wild type allele of the insertion site. In (B) a double homologous recombination event produces the recombinant directly. The open boxes represent flanking vaccinia virus DNA, the hatched box a vaccinia virus promoter, the solid line a foreign gene, the double line the virus genome, and the single line plasmid sequences.

Although the intermediate virus is unstable, it can be retained if the sequence between the direct repeats contains a dominant selectable maker for which selective pressure is maintained. This is the basis of a technique termed transient dominant selection, which enables mutations to be introduced into specific sites without leaving the genetic marker in the virus genome (41).

A plasmid is constructed that contains the desired mutated allele of the virus gene and distal to this a dominant selectable marker gene (for example *Ecogpt*) linked to a vaccinia virus promoter (*Figure 5*). Selection of recombinants in the presence of MPA (*Protocol 6*) ensures that all viruses have the entire plasmid integrated into the virus genome If selection is removed, unstable structures resolve and plaque isolates representing either parental or

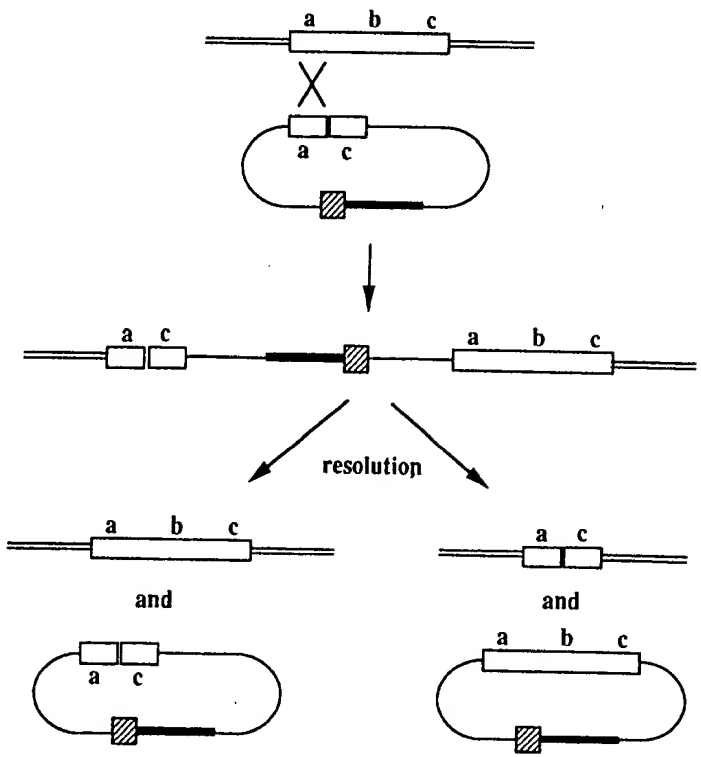

Figure 5. Formation of recombinants by transient dominant selection. The plasmid contains a vaccinia virus gene (open box) with an internal region ('b') deleted and a dominant selectable marker (solid line) such as *Ecogpt* linked to a vaccinia virus promoter (hatched box). Recombination with the homologous sequence 'a' of the virus genome produces an intermediate recombinant with direct repeats of sequences 'a' and 'c'. If selection for the marker gene is removed the intermediate virus resolves by recombination between repeated sequences 'a' or 'c'. If resolution occurs by recombination through sequence 'a' the WT virus genome and the plasmid that was used for transfection are formed. Alternatively, if recombination occurs between 'c' sequences the products are a recombinant with 'b' deleted and a plasmid bearing the complete vaccinia virus gene.

mutant virus can be distinguished by PCR using oligonucleotide primers that span the mutated site. If only WT virus plaques are obtained it is likely that the mutation is lethal for virus replication or plaque formation. The resolution event can be speeded by plaquing on *hprt*$^-$ cells and selecting against the presence of *Ecogpt* using 6-thioguanine (*Protocol 7*).

During resolution of the intermediate virus, plasmids are formed in addition to progeny virus genomes (42) (*Figures 4* and *5*). These represent either the original plasmid used to construct the recombinant, or a plasmid containing the WT allele rescued from the recombinant. Since all plasmids are replicated in vaccinia virus-infected cells, irrespective of whether they contain vaccinia virus sequences, plasmids formed during resolution events will be amplified and may then recombine with virus genomes causing virus heterogeneity (42). A screen for plasmid sequences should therefore be included during selection of recombinants to ensure that resolution has already occurred and that plasmid sequences do not persist. In addition, the plasmid should be digested prior to transfection with two restriction enzymes which produce incompatible termini in order to avoid formation of plasmids in virus-infected cells, since linear plasmids with compatible termini could be circularized by the virus DNA ligase. With this precaution, viable virus can only be produced by double homologous recombination.

4. Inducible gene expression from vaccinia virus vectors

Conventional vaccinia virus vectors allow the expression of a target gene at a time during infection and at a level determined by the selected vaccinia virus promoter. In the last few years, new vectors have been developed which allow genes to be regulated in an inducible manner in vaccinia virus-infected cells (29, 31, 43–46). As illustrated in *Figure 6*, these vectors use the *E. coli* lacI repressor protein and the operator sequence to which it binds. A chimeric gene is constructed which contains a late vaccinia virus promoter, one or two copies of the *lac* operator, and the open reading frame to be regulated. This gene is inserted into *tk* together with a constitutive vaccinia virus promoter driving the *lacI* ORF. The lacI protein binds to the operator and prevents transcription of the downstream ORF. This blockage may be released by the addition of isopropyl β-D-thiogalactopyranoside (IPTG), which causes the lacI protein to dissociate from the operator. The stringency of repression and degree of inducibility with IPTG depend upon the nature of the operator sequence, the proximity of the operator to the RNA start site of the vaccinia virus promoter, the number of operator copies, and the concentration of IPTG. One vector (pPR34) allows 97% inhibition of gene expression and 90% induction with 5 mM IPTG, and another (pPR35) has two copies of the operator and inhibits gene expression by 99.9% but permits only 50% induction (44).

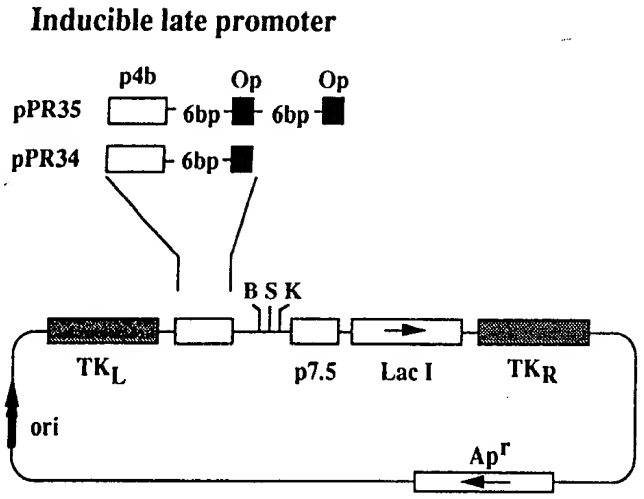

Figure 6. Plasmid vectors for IPTG-dependent gene expression from vaccinia virus. Plasmids pPR34 and pPR35 contain *lacI* downstream from the vaccinia virus 7.5 kDa promoter, the p4b promoter upstream of the *lac* operator, and flanking DNA from the left and right of *tk*. The plasmids differ only in the presence of a second copy of the operator sequence in pPR35. (This figure has been reproduced from ref. 44 with permission from Academic Press.)

These vectors potentially allow regulation of genes that would otherwise be toxic to vaccinia virus replication, and in addition have been most valuable for the study of vaccinia virus gene function. Two approaches have been taken. In one method, which is described in *Protocol 8* and illustrated in *Figure 7* (29, 31), a copy of the ORF to be studied is produced by PCR and cloned downstream from the IPTG-inducible promoter. This is inserted into vaccinia virus *tk* with *lacI* to form a *tk*$^-$ intermediate virus which contains an inducible copy of the ORF plus the endogenous copy. The endogenous copy is then disrupted by insertion of *Ecogpt* and a recombinant is selected in the presence of MPA and IPTG. This virus will have only a single inducible copy of the ORF and will be dependent on IPTG for replication if the ORF encodes an essential protein. To study the function of the protein during virus replication, a stock of virus is grown in the presence of IPTG and then used to infect cells in the absence of IPTG. The stage at which virus replication is aborted is then determined biochemically or by electron microscopy. This method may be used to study the function of any late gene, irrespective of whether a conditional lethal mutant exists, and has the advantage that the function of a protein with the WT amino acid sequence is studied rather than proteins bearing conditional lethal amino acid substitutions.

An alternative approach to studying vaccinia virus gene function using IPTG-inducible vectors uses the transient dominant selection method. First, a

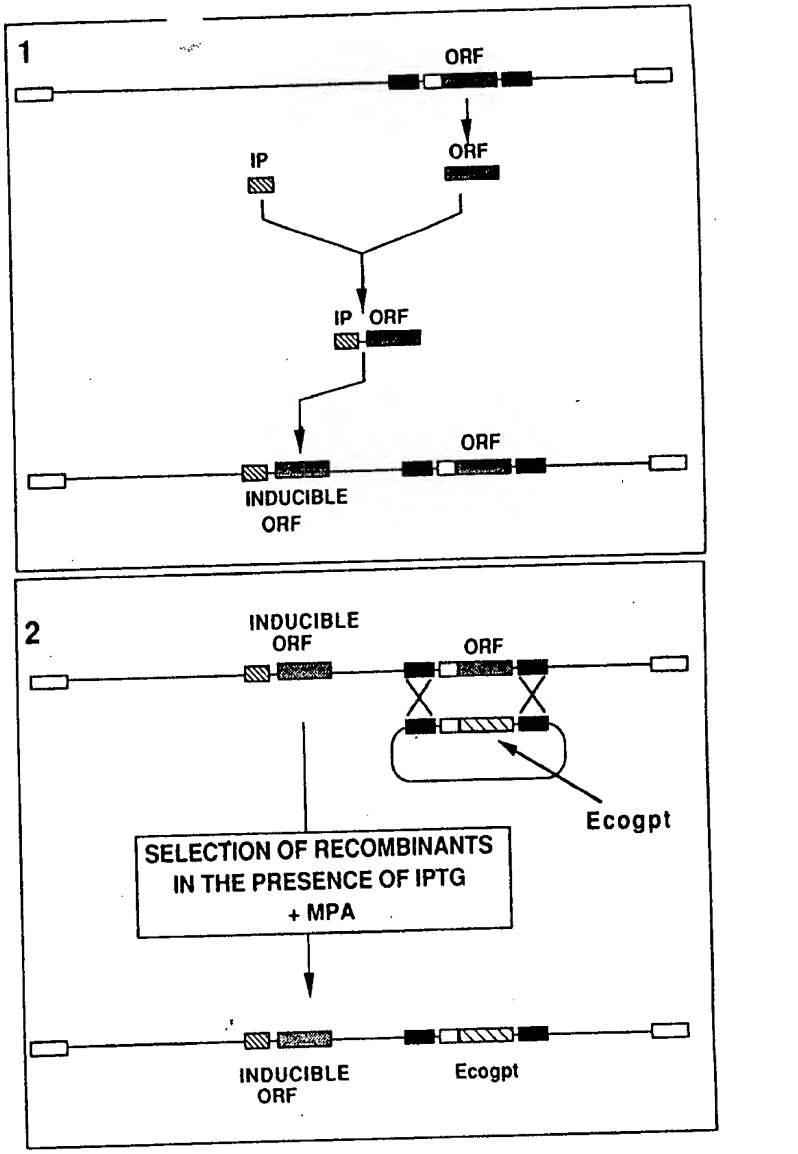

Figure 7. General scheme for the formation of IPTG-dependent vaccinia virus. (1) A PCR version of an ORF (stippled box) is cloned downstream of an IPTG-inducible promoter (hatched box) and then inserted into *tk* to form an intermediate virus. (2) Cells infected with the intermediate virus are transfected with a plasmid in which the ORF has been replaced by *Ecogpt* linked to a vaccinia virus promoter. Recombination between the vaccinia virus sequences flanking the ORF (filled box) forms a recombinant which is selected in the presence of MPA and IPTG.

virus is constructed that expresses the lacI protein from wit... *tk*. Next, the *lac* operator is inserted between the transcriptional initiation site of the gene to be regulated and the downstream ORF within a second plasmid vector. The *Ecogpt* gene linked to a constitutive vaccinia virus promoter is then cloned into this plasmid distal to the vaccinia virus gene. The resulting plasmid is transfected into cells infected with the virus expressing *lacI*, and recombinants are selected in the presence of MPA (*Protocol 6*). Progeny viruses retain the plasmid sequences and, after removal of MPA selection, resolve in the presence of IPTG to form either parental or recombinant virus, which may be distinguished by PCR across the site at which the operator has been inserted. If the gene being tested is required for replication or plaque formation, these events will be dependent upon IPTG.

Recombinant viruses which express IPTG-inducible copies of the 11 kDa core protein (gene *F18R*), the 14 kDa fusion protein (gene *A27L*), or a lectin-like glycoprotein (gene *SalL4R*) have been described. In the absence of IPTG the former virus produces no infectious progeny owing to a blockage in nucleoid condensation (47), while the latter two viruses produce normal amounts of INV but are dependent upon IPTG for plaque development and have altered EEV formation (29, 31).

To date, only vaccinia virus late genes have been regulated by the *lac* operator and lacI protein, as attempts to regulate early genes have not provided a sufficient degree of repression (J. F. Rodriguez, unpublished data). This presumably reflects the need to package lacI bound to the *lac* operator within all infectious virus particles, so that no expression occurs in freshly-infected cells without the addition of IPTG. Additionally, IPTG has to reach the virus genome within the core to induce efficiently. These problems may prevent efficient IPTG-inducible regulation of early genes, but adequate regulation of intermediate genes should be possible.

Protocol 8. Construction of IPTG-dependent vaccinia virus

Materials

- 0.1 M IPTG: filter-sterilize, aliquot, and store at −20°C

Method

1. Select the late gene to be regulated in an IPTG-inducible manner. Produce a copy of the protein-coding region by PCR and clone it downstream of the IPTG-inducible promoter in plasmid vector pPR34 or pPR35 (44). These vectors allow insertion of the foreign gene into *tk* together with *lacI* linked to the vaccinia virus 7.5 kDa promoter.
2. Infect CV-1 cells with WT vaccinia virus and transfect with the plasmid produced, as described in *Protocol 1*.
3. Isolate tk^- recombinants as described in *Protocol 2*.

4. Identify recombin... by PCR screen (*Protocol 4*).
5. Grow a stock of virus, titrate it, and infect CV-1 cells at an m.o.i. of 0.05 p.f.u./cell. Transfect the infected cells with a plasmid containing the target gene interrupted by *Ecogpt* linked to a vaccinia virus promoter (for example for interruption of the *SalL4R* gene use plasmid pSAD15: see ref. 31).
6. Select recombinants in the presence of MPA, xanthine, and hypoxanthine (*Protocol 6*) and 5 mM IPTG. Plaque purify the recombinants three times and grow a stock of virus in the presence of 5 mM IPTG.

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5. High-level gene expression

5.1 Powerful poxvirus promoters

Several approaches have been used to increase the level of expression from vaccinia virus expression vectors. Natural vaccinia virus promoters from genes encoding the most abundant late structural proteins, such as those encoding for 11 kDa and p4b, give levels of expression approximately three- to five-fold higher than the widely-used 7.5 kDa promoter. Synthetic promoters based upon the results of saturation mutagenesis of vaccinia virus promoters (48, 49) produce higher levels of expression than natural strong promoters (14). Promoters from other poxviruses which produce a cytoplasmic inclusion body composed predominantly of a single abundant virus protein, such as those from cowpox virus (50) and the *Choristoneura biennis* entomopoxvirus (51), are both highly active in vaccinia virus.

5.2 T7 RNA polymerase

The most successful method for high-level expression of foreign genes by vaccinia virus has utilized bacteriophage T7 RNA polymerase (52–56). The system is illustrated in *Figure 8*. Two recombinants are constructed, one which expresses the bacteriophage T7 RNA polymers from a vaccinia virus promoter, and a second which contains the gene for high-level expression under the control of T7 transcriptional initiation and termination sequences. Co-infection of cells with the two viruses enables expression of T7 RNA polymerase and high-level transcription of the target gene from the T7 RNA polymerase promoter. The transcripts are very abundant, representing 30% of total cellular RNA at 24 h p.i., but most are uncapped and therefore poorly translated in vaccinia virus-infected cells. This difficulty was overcome by insertion of the 5′ non-coding sequences of the picornavirus encephalomyocarditis virus, allowing cap-independent entry of ribosomes on to mRNA between the 5′ end of the T7 transcript and the ORF to be expressed. With this modification and the use of hypertonic medium to favour translation of virus mRNAs, chloramphenicol acetyltransferase was expressed at a level representing 10% of total cellular protein (54).

Although expression levels are highest if two recombinants are used, namely one that expresses T7 RNA polymerase and one containing the target gene, satisfactory levels of expression are obtained by infecting cells with the virus expressing T7 RNA polymerase and then transfecting them with a plasmid containing the target gene flanked by T7 RNA polymerase transcriptional control signals. This has the advantage of speed and is especially useful for screening multiple gene variants containing specific mutations. After selection of a suitable clone, the same plasmid can be used to construct a recombinant to achieve optimal levels of expression.

Further refinements in the expression system have been made by combining T7 RNA polymerase-based expression with IPTG-inducibility (56). Also, both the T7 RNA polymerase gene and the T7 promoter and termination sequences can be accommodated in a single virus, if expression of the polymerase is made IPTG-dependent (71). Previously, it had not been possible to have both components in the same virus, presumably because the very high level of transcription by T7 RNA polymerase prevented virus replication.

6. Safety considerations

Vaccinia virus is an established human vaccine but is also a class II human pathogen, and should be handled under category 2 laboratory containment.

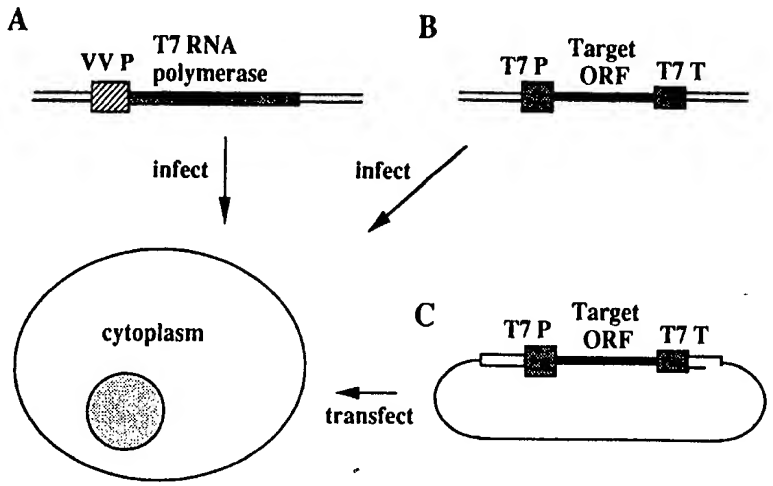

Figure 8. Expression of genes from vaccinia virus using bacteriophage T7 RNA polymerase. A virus (A) is constructed which contains the T7 RNA polymerase gene downstream from a vaccinia virus promoter (hatched box). A second recombinant (B) is formed which contains the target ORF (solid line) controlled by T7 promoter (T7 P) and terminator (T7 T) transcriptional sequences. Co-infection of cells with the two viruses allows high-level expression of the target ORF. For rapid screening of the target ORF, or variants of it, the plasmid (C) may be transfected into cells infected with virus (A).

Vaccination is no longer recommended in the UK unless the manipulation of the virus is considered likely to enhance its virulence, very large amounts of virus are being used, or the work involves animals. In the latter situation, a case-by-case evaluation of the need for vaccination should be undertaken. Vaccination is strictly contraindicated in potential vaccinees with eczema or any form of immunological deficiency.

Strains of vaccinia virus vary considerably in their virulence. For routine laboratory use, a virus with a deliberately engineered attenuating phenotype should be selected, such as one lacking *tk* (see also *Table 1*).

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References

1. Mackett, M., Smith, G. L., and Moss, B. (1985). In *DNA cloning: a practical approach* (ed. D. M. Glover), p. 191. IRL Press, Oxford.
2. Smith, G. L. and Mackett, M. (1992). In *Recombinant poxviruses* (ed. M. M. Binns and G. L. Smith), pp. 81–122. CRC Press, Boca Raton, Florida.
3. Moss, B. (1990). In *Virology* (ed. B. N. Fields and D. M. Knipe), p. 2079. Raven Press, New York.
4. Buller, R. M. L. and Palumbo, G. J. (1991). *Microbiol. Rev.*, **55**, 80.
5. Moss, B. (1990). *Annu. Rev. Biochem.*, **59**, 661.
6. Traktman, P. (1990). *Curr. Top. Microbiol. Immunol.*, **163**, 93.
7. Payne, L. G. (1980). *J. Gen. Virol.*, **50**, 89.
8. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). *Virology*, **179**, 247.
9. Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Norton, E. K., and Paoletti, E. (1991). *Virology*, **180**, 406.
10. Keck, J. G., Baldick, C. J., and Moss, B. (1990). *Cell*, **61**, 801.
11. Kent, R. K. (1988). Isolation and analysis of the vaccinia virus p4b gene promoter. PhD thesis, University of Cambridge, UK.
12. Mackett, M., Smith, G. L., and Moss, B. (1984). *J. Virol.*, **49**, 857.
13. Chakrabarti, S., Brechling, K., and Moss, B. (1985). *Mol. Cell. Biol.*, **5**, 3403.
14. Davison, A. J. and Moss, B. (1990). *Nucl. Acids Res.*, **18**, 4285.
15. Falkner, F. G. and Moss, B. (1988). *J. Virol.*, **62**, 1849.
16. Perkus, M. E., Limbach, K., and Paoletti, E. (1989). *J. Virol.*, **63**, 3829.
17. Bertholet, C., Drillien, R., and Wittek, R. (1985). *Proc. Natl Acad. Sci. USA*, **82**, 2096.
18. Rosel, J. L. and Moss, B. (1985). *J. Virol.*, **56**, 830.
19. Browne, H., Churcher, M., Stanley, M., Smith, G. L., and Minson, A. C. (1988). *J. Gen. Virol.*, **69**, 1263.
20. Earl, P. L., Hugin, A. W., and Moss, B. (1990). *J. Virol.*, **64**, 2448.
21. Coupar, B. E. H., Andrew, M. E., Both, G. W., and Boyle, D. B. (1986). *Eur. J. Immunol.*, **16**, 1479.

22. Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, A., Boyle, D. B., Chan, Y., and Smith, G. (1988). *J. Exp. Med.*, **168**, 1211.
23. Mackett, M., Smith, G. L., and Moss, B. (1982). *Proc. Natl Acad Sci. USA*, **79**, 7415.
24. Buller, R. M. L., Smith, G. L., Cremer, K., Notkins, A. L., and Moss, B. (1985). *Nature*, **317**, 813.
25. Buller, R. M. L., Chakrabarti, S., Cooper, J. A., Twardzik, D. R., and Moss, B. (1988). *J. Virol.*, **62**, 866.
26. Kotwal, G. J., Hugin, A. W., and Moss, B. (1989). *Virology*, **171**, 579.
27. Kotwal, G. J., Isaacs, S. N., McKenzie, R., Frank, M. M., and Moss, B. (1990). *Science*, **250**, 827.
28. Moore, J. B. and Smith, G. L. (1992). *EMBO J.*, **11**, 1973.
29. Rodriguez, J. F. and Smith, G. L. (1990). *Nucl. Acids Res.*, **18**, 5347.
30. Blasco, R. and Moss, B. (1991). *J. Virol.*, **65**, 5910.
31. Duncan, S. A. and Smith, G. L. (1992). *J. Virol.*, **66**, 1610.
32. Panicali, D., Grzelecki, A., and Huang, C. (1986). *Gene*, **47**, 193.
33. Ramshaw, I. A., Andrew, M. E., Philips, S. M., Boyle, D. B., and Coupar, B. E. H. (1987). *Nature*, **329**, 545.
34. Franke, C. A., Rice, C. M., Strauss, J. H., and Hruby, D. E. (1985). *Mol. Cell. Biol.*, **5**, 1918.
35. Boyle, D. B. and Coupar, B. E. H. (1988). *Gene*, **65**, 123.
36. Isaacs, S. N., Kotwal, G. J., and Moss, B. (1990). *Virology*, **178**, 626.
37. Wiktor, T. J., Macfarlan, R. I., Reagan, K. J., Dietzschold, B., Curtis, P. J., Wunner, W. H., Kieny, M.-P., Lathe, R., Lecocq, J.-P., Mackett, M., Moss, B., and Koprowski, H. (1984). *Proc. Natl Acad. Sci. USA*, **81**, 7194.
38. Fathi, Z., Sridhar, P., Pacha, R. F., and Condit, R. C. (1986). *Virology*, **155**, 97.
39. Ball, L. A. (1987). *J. Virol.*, **61**, 1788.
40. Spyropoulos, D. D., Roberts, B. E., Panicali, D., and Cohen, L. K. (1988). *J. Virol.*, **62**, 1046.
41. Falkner, F. G. and Moss, B. (1990). *J. Virol.*, **64**, 3108.
42. Kerr, S. M. and Smith, G. L. (1991). *Virology*, **180**, 625.
43. Fuerst, T. R., Fernandez, M. P., and Moss, B. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 2549.
44. Rodriguez, J. F. and Smith, G. L. (1990). *Virology*, **177**, 239.
45. Zhang, Y. and Moss, B. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 1511.
46. Zhang, Y. and Moss, B. (1991). *J. Virol.*, **65**, 6101.
47. Blasco, R., Cole, N. B., and Moss, B. (1991). *J. Virol.*, **65**, 4598.
48. Davison, A. J. and Moss, B. (1989). *J. Mol. Biol.*, **210**, 771.
49. Davison, A. J. and Moss, B. (1989). *J. Mol. Biol.*, **210**, 749.
50. Patel, D. D., Ray, C. A., Drucker, R. P., and Pickup, D. J. (1988). *Proc. Natl Acad. Sci. USA*, **85**, 9431.
51. Pearson, A., Richardson, C., and Yuen, L. (1991). *Virology*, **180**, 561.
52. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 8122.
53. Fuerst, T. R., Earl, P. L., and Moss, B. (1987). *Mol. Cell. Biol.*, **7**, 2538.
54. Elroy-Stein, O., Fuerst, T. F., and Moss, B. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 6126.
55. Fuerst, T. R. and Moss, B. (1989). *J. Mol. Biol.*, **206**, 333.

56. Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A., and Fuerst, T. (1990). *Nature*, **348**, 91.
57. Patel, A. H., Gaffney, D. F., Subak-Sharpe, J. H., and Stow, N. D. (1990). *J. Gen. Virol.*, **71**, 2013.
58. Perkus, M. E., Panicali, D., Mercer, S., and Paoletti, E. (1986). *Virology*, **152**, 285.
59. Law, K. M. and Smith, G. L. (1992). *J. Gen. Virol.*, **73**, 549.
60. Beattie, E., Tartaglia, J., and Paoletti, E. (1991). *Virology*, **183**, 419.
61. Child, S. J., Palumbo, G. J., Buller, R. M. L., and Hruby, D. E. (1990). *Virology*, **174**, 625.
62. Niles, E. G. and Seto, J. (1988). *J. Virol.*, **62**, 3772.
63. Duncan, S. A. and Smith, G. L. (1992). *J. Gen. Virol.*, **73**, 1235.
64. Hughes, S. J., Johnston, L. H., de Carlos, A., and Smith, G. L. (1991). *J. Biol. Chem.*, **266**, 20103.
65. Colinas, R. J., Goebel, S. J., Davis, S. W., Johnson, G., Norton, E. K., and Paoletti, E. (1990). *Virology*, **179**, 267.
66. Shida, H., Tochikura, T., Sato, T., Konno, T., Hirayoshi, K., Seki, M., Ito, Y., Hatanaka, M., Hinuma, Y., Sugimoto, M., Takahashi-Nishimaki, F., Maruyama, T., Miki, K., Suzuki, K., Morita, M., Sashiyama, H., and Hayami, M. (1987). *EMBO J.*, **6**, 3379.
67. Shida, H., Hinuma, Y., Hatanaka, M., Morita, M., Kidokoro, M., Suzuki, K., Maruyama, T., Takahashi-Nishimaki, F., Sugimoto, M., Kitamura, R., Miyazawa, T., and Hayami, M. (1988). *J. Virol.*, **62**, 4474.
68. Takahashi-Nishimaki, F., Funahashi, S.-I., Miki, K., Hashizume, S., and Sugimoto, M. (1991). *Virology*, **181**, 158.
69. Zhou, J., McLean, L., Sun, X.-Y., Stanley, M., Almond, N., Crawford, L., and Smith, G. L. (1990). *J. Gen. Virol.*, **71**, 2185.
70. Smith, G. L., Chan, Y. S., and Howard, S. T. (1991). *J. Gen. Virol.*, **72**, 1349.
71. Alexander, W. A., Moss, B., and Fuerst, T. R. (1992). *J. Virol.*, **66**, 2934.

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The Complete Genomic Sequence of the Modified Vaccinia Ankara Strain: Comparison with Other Orthopoxviruses

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The complete genomic DNA sequence of the highly attenuated vaccinia strain modified vaccinia Ankara (MVA) was determined. The genome of MVA is 178 kb in length, significantly smaller than that of the vaccinia Copenhagen genome, which is 192 kb. The 193 open reading frames (ORFs) mapped in the MVA genome probably correspond to 177 genes, 25 of which are split and/or have suffered mutations resulting in truncated proteins. The left terminal genomic region of MVA contains four large deletions and one large insertion relative to the Copenhagen strain. In addition, many ORFs in this region are fragmented, leaving only eight genes structurally intact and therefore presumably functional. The inserted DNA codes for a cluster of genes that is also found in the vaccinia WR strain and in cowpox virus and includes a highly fragmented gene homologous to the cowpox virus host range gene, providing further evidence that a cowpox-like virus was the ancestor of vaccinia. Surprisingly, the central conserved region of the genome also contains some fragmented genes, including ORF F5L, encoding a major membrane protein, and ORFs F11L and O1L encoding proteins of 39.7 and 77.6 kDa, respectively. The right terminal genomic region carries three large deletions: all classical poxviral immune evasion genes and all ankyrin-like genes located in this region are fragmented except for those encoding the interleukin-1β receptor and the 68-kDa ankyrin-like protein B18R. Thus, the attenuated phenotype of MVA is the result of numerous mutations, particularly affecting the host interactive proteins, including the ankyrin-like genes, but also involving some structural proteins. © 1998 Academic Press

INTRODUCTION

The members of the poxvirus family have large double-stranded DNA genomes encoding several hundred proteins (review: Moss, 1996). Several members of the poxviridae have been sequenced recently, including the vaccinia virus Copenhagen (CPN) strain (Goebel *et al.*, 1990) encompassing 192 kb, the variola (VAR) strains Bangladesh (Massung *et al.*, 1994) and India (Shchelkunov *et al.*, 1993d) encompassing 186 kb, and the human tumorigenic poxvirus molluscum contagiosum virus (Senkevich *et al.*, 1996) encompassing 190 kb. Sequence analysis of poxvirus genomes has increased our knowledge of the structure and function of poxviral genes and increased our understanding of host–virus interactions. Due to the complexity of poxviral genomes and the complex viral life cycle, questions concerning immunogenicity, virulence, and host range of poxvirus strains have been answered only partially for the respective virus strain. It was therefore of interest to determine the genomic sequence of the highly attenuated vaccinia strain modified vaccinia Ankara (MVA) (Mayr *et al.*, 1978), which cannot grow in most mammalian cells and which is a good candidate for a recombinant vaccine vector (Sutter and Moss, 1992; Sutter *et al.*, 1994). This strain has been passaged over 570 times in chicken embryo fibroblasts, during which six major deletions relative to the parental wild-type strain Ankara, accompanied by a severe restriction in host range, have occurred (Meyer *et al.*, 1991). Precise restriction maps have been established (Meyer *et al.*, 1991) and two of the deletions have been characterized by sequence analysis (Altenburger *et al.*, 1989; Antoine *et al.*, 1996). In a first step to elucidate the genetic basis for the high degree of attenuation of MVA, the nucleotide sequence of its genomic DNA was determined and the open reading frames were compared to the entries of current sequence databases.

RESULTS

Basic genome data

The sequence of the linear, double-stranded DNA molecule totals 177,923 bp and the G+C content is 33.4%, exactly corresponding to the G+C content of the CPN strain. Translational analysis allowed the mapping of 192 ORFs specifying potential proteins ≥65 amino acids. As in other orthopoxviruses, the ORF encoding the RNA polymerase subunit rpo7, G5.5R (MVA083R), is 63 amino acids in size (Amegadzie *et al.*, 1992). Eight duplicated ORFs ≥65 amino acids that are located in the repeat regions within the terminal 5.5 kb of the inverted terminal repeats (ITRs) were not listed in Table 1 because they presumably do not represent functional genes. Translation of these ORFs resulted in a family of related proteins with repeat

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TABLE 1

Features and Homologies of Open Reading Frames of the Vaccinia MVA Strain

ORF[a] left	START / STOP terminal	AA[b] region:	kDa[c]	name / (putative) function / homologies[d]	BLAST[e] expect	BLAST[f] AA id	HSS[g] (%)	references
001L/	6822	136	14.9	35k major secr. protein				(Patel *et al.*, 1990)
193R[h]	6412			chemokine receptor (f')				(Graham *et al.*, 1997)
C23L		244		VAC (*C23L/B29R*)	6.0e-57	41/42	97	(Goebel *et al.*, 1990)
		253		VAR-I G3R	8.9e-51	46/49	93	(Shchelkunov *et al.*, 1995)
		246		CPX ORF B	5.6e-49	40/42	95	(Hu *et al.*, 1994)
		258		SFV T1 protein	2.5e-20	23/42	54	(Upton *et al.*, 1987)
		260		Myxoma virus T1/35kDa	1.5e-14	21/42	50	(Graham *et al.*, 1997)
002L/	7784	176	19.7	secr. TNF receptor (f)				
192R[h]	7254	355		CPX crmB	5.1e-71	76/83	91	(Upton *et al.*, 1991a)
		348		VAR-BSH G2R	1.0e-66	73/83	87	(Hu *et al.*, 1994)
		326		Myxoma virus T2	4.9e-30	21/37	56	(Shchelkunov *et al.*, 1995)
		325		Rabbit fibroma Virus T2	1.8e-28	17/36	47	(Upton *et al.*, 1991a)
		202		CPX C4L	8.7e-15	30/51	58	(Upton *et al.*, 1987)
		346		'HS TNF receptor protein	1.9e-08	14/26	53	(Safronov *et al.*, 1996)
C19L		259		VAC (*C19L/B25R*)	0.00026	16/19	84	(Heller *et al.*, 1990)
		277		human CD40L receptor	0.0015	11/24	45	(Goebel *et al.*, 1990)
				30 matches to TNF receptors and surface proteins	<0.39			(Stamencovic *et al.*, 1989)
003L/	8780	102	12.1	45k ank[i]-like protein (f1)				
191R[h]	8472			VAC C17L/B23R				(Goebel *et al.*, 1990)
C17L		386			1.3e-39	62/63	98	
004L/	9558	233	26.9	45k ank-like protein (f2)				(Goebel *et al.*, 1990)
190R[h]	8857			VAC (C17L/B23R)				(Goebel *et al.*, 1990)
C17L		386		VAR-BSH	6.2e-159	110/110	100	
D1L		91		CPX host range	9.1e-31	46/49	93	(Goebel *et al.*, 1990)
		669		VAR-I D6L (BSH:D8L)	1.1e-13	22/50	44	(Shchelkunov *et al.*, 1995)
		452		VAR-I B19R (BSH: B16R)	1.7e-11	21/50	42	(Spehner *et al.*, 1988)
		574		VAC B18R (WR: B17R)	1.2e-05	22/73	30	(Shchelkunov *et al.*, 1995)
		574		VAC C9L	8.6e-05	22/73	30	(Shchelkunov *et al.*, 1995)
		634		VAR-I G1R	0.00011	11/24	45	(Goebel *et al.*, 1990)
		585		orf virus	0.00013	22/74	29	(Kotwal and Moss, 1988a)
		516		VAR-I D7L (BSH:D10L)	0.0088	15/49	30	(Shchelkunov *et al.*, 1995)
		153			0.014	12/28	42	(Sullivan *et al.*, 1995b)
								(Shchelkunov *et al.*, 1995)
005R	10203	140	15.5	Growth factor (EGF receptor binding)				
	10625			VAC				(Twardzik *et al.*, 1985)
C11R		142		VAR-I (BSH:D4R)	2.9e-82	99/104	95	(Stroobant *et al.*, 1985)
D2R		140		CPX D5R	3.6e-74	106/140	75	(Goebel *et al.*, 1990)
		138		human epiregulin	3.4e-95	101/114	88	(Shchelkunov *et al.*, 1995)
		169		100 matches to growth factor like sequences	2.2e-14	29/78	37	(Safronov *et al.*, 1996)
					<0.10			D30783
006L	11758	326	37.9	37.9k protein				
C10L	10778	331		VAC				(Venkatesan *et al.*, 1982)
		331		CPX D6L	1.7e-235	264/268	98	(Goebel *et al.*, 1990)
D5L		330		VAR-BSH (I: D3L)	7.7e-235	264/268	98	(Safronov *et al.*, 1996)
		316		VAR-I D11L (BSH:D14L)	3.6e-233	169/171	97	(Shchelkunov *et al.*, 1995)
		316		VAC C4L	1.7e-94	34/68	44	(Shchelkunov *et al.*, 1995)
		315		CPX D16L	1.8e-92	30/68	54	(Goebel *et al.*, 1990)
		82		Ectromelia 42K protein	2.3e-92	31/68	45	(Safronov *et al.*, 1996)
		418		FPV BamHI ORF1	1.2e-50	78/82	95	(Senkevich *et al.*, 1993a)
					3.0e-11	13/41	31	(Tomley *et al.*, 1988)
007R	12263	91	10.6	28k virulence factor (f)				
	12538	242		CPX D7R	1.5e-51	42/47	89	(Senkevich *et al.*, 1993a)
		184		VAC-WR 21.7k protein	5.3e-51	41/47	87	(Safronov *et al.*, 1996)
D4R		242		VAR-I (BSH:D6R)	3.7e-50	41/47	87	(Kotwal and Moss, 1988a)
		241		Ectromelia 28k secreted virulence factor	3.7e-50	41/47	87	(Shchelkunov *et al.*, 1995)
								(Senkevich *et al.*, 1993a)
008L	13414	120	13.7	13.7k protein				
D7L	13052	126		VAR-BSH (I:D5L)	1.9e-83	57/64	89	(Shchelkunov *et al.*, 1995)
		138		Ectromelia 16k protein	7.8e-81	58/60	96	(Senkevich *et al.*, 1993a)
		124		CPX D8L	3.2e-67	49/60	81	(Safronov *et al.*, 1996)
		68		7.8k protein (VAC-WR)	1.3e-34	53/64	82	(Kotwal and Moss, 1988a)
009L	13745	90	10.7	77k CPX hr protein (f1)				
	13473	669		CPX host range gene	2.7e-46	43/52	82	(Spehner *et al.*, 1988)
		634		VAC C9L	1.7e-05	9/33	27	(Safronov *et al.*, 1996)
010L	14186	142	16.1	77k CPX hr protein (f2)				(Goebel *et al.*, 1990)
	13758	669		CPX host range gene	2.2e-91	133/142	93	(Spehner *et al.*, 1988)
		634		VAC C9L	9.2e-21	26/63	41	(Safronov *et al.*, 1996)
D6L		452		VAR-I (BSH: D8L)	4.5e-13	27/29	93	(Goebel *et al.*, 1990)
		150		VAC C18L/B24R	1.3e-11	19/52	36	(Shchelkunov *et al.*, 1995)
		439		AT ankyrin repeat protein	9.5e-07	23/59	38	(Goebel *et al.*, 1990)
		558		VAR-I B6R (BSH:B5R)	4.0e-05	28/113	24	(Zhang *et al.*, 1992)
				30 matches with ankyrin repeat containing proteins	2.7e-05 to 0.016			(Shchelkunov *et al.*, 1995)
011L	14682	135	15.8	77k CPX hr protein (f3)				
	14275	669		CPX host range gene	7.6e-80	54/64	84	(Spehner *et al.*, 1988)
D6L		452		VAR-I (BSH: D8L)	9.2e-78	52/64	81	(Safronov *et al.*, 1996)
012L	15183	90	10.3	77k CPX hr protein (f4)				(Shchelkunov *et al.*, 1995)
								(Spehner *et al.*, 1988)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[a] region:	kDa[a]	name / (putative) function / homologies[a]	BLAST[a] expect	BLAST[a] AA id	HSS[a] (%)	references
D6L	14911	452		VAR-I (BSH: D8L)	2.2e-52	80/85	94	(Shchelkunov *et al.*, 1995)
		669		CPX host range gene	8.1e-51	77/85	90	(Spehner *et al.*, 1988)
		153		VAR-I D7L (BSH: D10L)	2.9e-17	19/45	42	(Shchelkunov *et al.*, 1995)
		634		VAC C9L	1.3e-13	19/45	42	(Goebel *et al.*, 1990)
		1161		C. botulinum NTNH protein	0.00019	6/12	50	(Hutson *et al.*, 1996)
		202		Capripox	0.00058	15/58	25	(Cao *et al.*, 1995)
		895		UDP glucose dehydrogenase	0.00051	6/19	31	(Bult *et al.*, 1996)
		516		orf virus ank-like	0.0064	16/49	32	(Sullivan *et al.*, 1995b)
		673		rabbit fibroma 77.2k protein	0.0072	12/30	40	(Massung *et al.*, 1992)
013L	15420	71	8.5	77k CPX hr protein (f5)				
	15205	669		CPX host range gene	5.2e-44	68/69	98	(Spehner *et al.*, 1988)
D6L		452		VAR (BSH: D8L)	7.9e-42	64/67	95	(Safronov *et al.*, 1996)
		673		rabbit fibroma 77.2k protein	0.0052	8/26	30	(Shchelkunov *et al.*, 1995)
		386		VAC C17L/B23R	0.018	14/33	42	(Massung *et al.*, 1992)
		202		Capripox	0.023	10/19	52	(Goebel *et al.*, 1990)
		574		VAC B18R (WR: B17R)	0.71	12/28	42	(Sullivan *et al.*, 1995b)
		574		VAR B19R (BSH:B16R)	0.71	12/28	42	(Goebel *et al.*, 1990)
								(Shchelkunov *et al.*, 1995)
014L	16205	109	13.1	75k ank-like gene (f1)				
C9L	15876	634		VAC	3.9e-73	109/109	100	(Kotwal and Moss, 1988a)
		614		CPX D11L	1.6e-70	105/108	97	(Goebel *et al.*, 1990)
D9L		91		VAR (I: D6.5L)	1.2e-52	78/91	85	(Safronov *et al.*, 1996)
		437		CPX D1L	3.7e-19	28/67	41	(Shchelkunov *et al.*, 1995)
		673		rabbit fibroma 77.2K protein	0.021	5/16	31	(Safronov *et al.*, 1996)
015L	16786	96	11.2	75k ank-like gene (f2)				(Massung *et al.*, 1992)
C9L	16496	634		VAC	4.0e-53	80/80	100	(Kotwal and Moss, 1988a)
		614		CPX D11L	3.9e-25	48/80	60	(Goebel *et al.*, 1990)
		437		CPX D1L	9.6e-12	14/36	38	(Safronov *et al.*, 1996)
		172		VAR-Garcia 1966 B11L	0.0001	17/17	100	(Safronov *et al.*, 1996)
		141		integrase (simian foamy v.)	0.033	10/24	41	(Massung *et al.*, 1996)
		669		CPX host range gene	0.043	9/17	52	(Schweizer and Neumann, 1995)
016L	17759	297	35.0	75k ank-like gene (f3)				(Spehner *et al.*, 1988)
C9L	16866	634		VAC	3.4e-208	291/294	98	(Kotwal and Moss, 1988a)
		614		CPX D11L	1.4e-130	90/126	71	(Goebel *et al.*, 1990)
D7L		153		VAR-I (BSH:D10L)	8.4e-68	84/109	77	(Safronov *et al.*, 1996)
		669		CPX host range gene	4.5e-17	24/61	39	(Shchelkunov *et al.*, 1995)
		452		CPX D9L	2.2e-16	23/61	37	(Spehner *et al.*, 1988)
D8L		668		VAR-BSH (I:D6L)	3.3e-16	21/61	34	(Safronov *et al.*, 1996)
		386		VAC C17L/B23R	2.9e-08	11/24	45	(Shchelkunov *et al.*, 1995)
		833		CPX D3L	0.0085	13/58	22	(Goebel *et al.*, 1990)
		574		VAC B18R (WR:B17R)	0.012	13/40	32	(Safronov *et al.*, 1996)
		202		Capripox virus	0.084	11/29	37	(Goebel *et al.*, 1990)
		574		VAR-I B19R (BSH:B16R)	0.090	13/40	32	(Sullivan *et al.*, 1995b)
								(Shchelkunov *et al.*, 1995)
017L	18335	177	20.8	20.8k protein				
C8L	17802	184		VAC	1.2e-125	125/129	96	(Kotwal and Moss, 1988a)
		182		CPX D12L	5.0e-118	119/126	94	(Goebel *et al.*, 1990)
		182		VAC B7R	8.3e-06	16/67	23	(Safronov *et al.*, 1996)
		795		VAC H4L (RAP94)	0.60	12/45	26	(Goebel *et al.*, 1990)
								(Goebel *et al.*, 1990)
018L	18859	150	18.0	host range protein				
C7L	18407	150		VAC	1.6e-106	150/150	100	(Perkus *et al.*, 1991)
D11L		150		VAR-BSH (I:D8L)	4.2e-106	149/150	99	(Kotwal and Moss, 1988a)
		185		Swinepox virus ORF SwF8a	3.4e-35	31/82	37	(Shchelkunov *et al.*, 1995)
		197		Capripox virus ORF CF8a	1.4e-31	29/87	33	(Schnitzlein and Tripathy, 1991)
		170		CPX D4L	3.5e-17	19/60	31	(Gershon and Black, 1989a)
		158		Myxoma virus ORF MF8	5.6e-13	16/43	37	(Safronov *et al.*, 1996)
		128		VAR-BSH D3L (I:D1.5L)	5.4e-06	18/60	30	(Jackson and Bults, 1992)
								(Shchelkunov *et al.*, 1995)
019L	19541	157	18.2	18.2k protein				
C6L	19068	151		VAC	7.6e-104	151/151	100	(Kotwal and Moss, 1988a)
D9L		156		VAR (BSH: D12L)	1.6e-99	145/150	96	(Goebel *et al.*, 1990)
		156		CPX D14L	1.3e-96	141/150	94	(Shchelkunov *et al.*, 1995)
		159		Capripox virus ORF T3a	4.4e-07	24/76	31	(Safronov *et al.*, 1996)
		151		Rabbit fibroma virus T3Aa	0.0047	16/46	34	(Gershon and Black, 1989a)
		181		VAC C16L/B22R	0.2	12/46	26	(Upton *et al.*, 1987)
		149		VAR C4R	0.29	8/13	61	(Goebel *et al.*, 1990)
		149		VAC-WR K7R	0.40	8/13	61	(Shchelkunov *et al.*, 1995)
								(Kotwal and Moss, 1988a)
020L	20025	113	13.2	14k virulence factor,				
	19684			secreted protein (f)				(Kotwal and Moss, 1988a)
N1L		117		VAC	2.6e-60	92/102	90	(Kotwal and Moss, 1988b)
		117		CPX P1L	7.3e-58	85/102	83	(Goebel *et al.*, 1990)
P1L		117		VAR-BSH, virokine	6.6e-56	88/102	86	(Shchelkunov *et al.*, 1995)
		107		Rabbit fibroma virus	0.015	10/17	58	(Safronov *et al.*, 1996)
								(Massung *et al.*, 1992)
021L	20656	170	20.3	alpha-amanitin sensitive				
	20144			protein				(Tamin *et al.*, 1991)
		175		CPX P2L	3.0e-118	138/142	97	(Kotwal and Moss, 1988a)
N2L		175		VAC	6.1e-116	137/142	96	(Safronov *et al.*, 1996)
P2L		177		VAR	9.7e-115	135/142	95	(Goebel *et al.*, 1990)
								(Shchelkunov *et al.*, 1995)
022L	20981	98	11.0	33k host range gene (f)				
K1L	20685	284		VAC	1.8e-56	86/88	97	(Gillard *et al.*, 1986)
		284		CPX M1L	2.3e-56	86/88	97	(Altenburger *et al.*, 1989)
C1L		66		VAR	2.0e-39	63/66	95	(Safronov *et al.*, 1996)
		65		human NOTCH 2	0.00036	17/41	41	(Shchelkunov *et al.*, 1995)
								(Kaisanis *et al.*, 1996)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[a]	kDa[a]	name / (putative) function / homologies[a]	BLAST[d] expect	BLAST[e] AA id		HSS[f] (G)	references
left terminal region:									
023L	22296 21187	369	42.3	serpin SPI-3, cell-cell fusion mutation					(Boursnell *et al.*, 1988)
K2L		369		VAC	1.2e-258	365/369	98		(Altenburger *et al.*, 1989)
C2L		373		CPX M2L	1.2e-256	331/337			(Goebel *et al.*, 1990)]
		373		VAR-BSH	9.9e-249	321/337	95		(Safronov *et al.*, 1996)
		373		Ectromelia virus H14-B	6.5e-244	312/337			(Shchelkunov *et al.*, 1995)
		386		HS plasminogen activator inhibitor 1	1.1e-35	30/68	44		U67964 (Loskutoff *et al.*, 1987)
		58		CPX SPI 3 protein	8.2e-33	57/58	98		gi:1168082
		369		Myxoma virus MAP1 gene	7.3e-32	33/131	25		(Upton *et al.*, 1990a)
		397		mouse protease nexin	1.5e-29	31/67	46		(Vassalli *et al.*, 1993)
		397		humane glia derived neurite-promoting factor	8.7e-27	30/65	46		A03911
		320		Swinepox SPI like protein	3.6e-21	20/70	28		(Massung *et al.*, 1993)
		417		a-1 antitrypsin, human	2.2e-20	26/66	39		(Ciliberto *et al.*, 1985)
		383		Corticosteroid-binding protein (rabbit)	9.0e-20				(Seralini *et al.*, 1989)
		390		squamous cell carcinoma antigen	1.9e-17				(Schneider *et al.*, 1995)
024L	22612 22346	88	10.5	IFN resistance, eIF-2a homolog					(Beattie *et al.*, 1991)
		88		CPX M3L	2.6e-61	88/88	100		(Davies *et al.*, 1992)
K3L		88		VAC	1.4e-60	87/88	98		(Safronov *et al.*, 1996)
C3L		88		VAR-1	1.0e-52	73/88	82		(Goebel *et al.*, 1990)
		86		SPV C8 protein	4.1e-22	20/44	45		(Shchelkunov *et al.*, 1995)
				translation initiation factor 2 family	1.2e-08/ 0.45				(Massung *et al.*, 1993)
025L	23938 22664	424	48.9	phospholipase D-like protein					(Cao *et al.*, 1997)
K4L		424		VAC	1.5e-306	423/424	99		(Goebel *et al.*, 1990)
		424		CPX M4L	2.1e-303	416/424	98		(Safronov *et al.*, 1996)
		437		human HU-K4	2.8e-135	53/95	55		U60644
		372		D. discoideum	2.5e-91	28/47	59		(Giorda *et al.*, 1989)
		516		C. elegans	6.6e-89	31/61	50		gi: 2435624
		2327		C. elegans	2.8e-52	36/60	60		gi: 2291241
		635		C. elegans	1.1e-24	19/53	35		(Wilson *et al.*, 1994)
		377		FPV major envelope protein	2.9e-23	19/61	31		(Calvert *et al.*, 1992)
		371		Myxoma virus env protein	3.6e-22	18/51	35		U43549
		378		Orf virus env protein B2L	1.2e-21	21/71	29		(Sullivan *et al.*, 1994)
MC021L		388		MCV subtype 1 env protein	3.2e-21	20/63	31		(Senkevich *et al.*, 1997)
C17L		372		VAR-BSH	4.6e-19	15/52	28		(Shchelkunov *et al.*, 1995)
		372		VAC F13L	4.9e-17	15/52	28		(Goebel *et al.*, 1990)
026L	24478 23966	170	19.1	lysophospholipase-like protein (f1)					(Upton & Buller, unpub.)
		276		CPX M5L	2.6e-110	161/170	94		(Safronov *et al.*, 1996)
K5L		277		Ectromelia virus H14-E	2.7e-109	160/170	94		X94355 U67964
		136		VAC	5.5e-69	107/108	99		(Goebel *et al.*, 1990)
		134		VAC-WR	8.3e-63	98/101	97		(Boursnell *et al.*, 1988)
		313		HS lysophospholipase homolog	3.3e-35	35/105	33		U67963
		323		poss. oxidoreductase M. tuberculosum	1.2e-13	30/94	31		Z97050
		324		Lysophospholipase isolog A. thaliana	3.1e-5	13/58	22		U95973
		313		H. influenza probable lysophospholipase L2	0.047	13/30	43		U32747
027L	24694 24500	64	7.0	lysophospholipase-like protein (f2)					(Upton & Buller, unpub.)
K6L		81		VAC	5.3e-42	63/63	100		(Boursnell *et al.*, 1988)
		276		CPX M5L	2.4e-36	57/58	98		(Safronov *et al.*, 1996)
		277		Ectromelia virus H14-E	2.4e-36	57/58	98		U67964
		313		HS lyophospholipase homolog	9.1e-23	34/53	64		U67963
		323		hyp. oxidoreductase M. tuberculosis	9.9e-14	22/54	40		Z97050
		530		dihydrotestosterone/androstanediol UDP-glucuronosyl-transferase	7.9e-05	6/17	35		A48633
central conserved region:									
028R	24864	149	17.5	17.5k protein					(Goebel *et al.*, 1990)
K7R	25313	149		VAC	6.1e-105	149/149	100		(Goebel *et al.*, 1990)
		161		CPX M6R	1.6e-101	144/149	96		(Safronov *et al.*, 1996)
C4R		149		VAR	4.9e-101	143/149	100		(Shchelkunov *et al.*, 1995)
		236		Swinepox (sc76)	0.00017	19/49	95		(Massung *et al.*, 1993)
029L	26046	222	25.9	25.9k protein					(Roseman and Slabaugh, 1990)
F1L	25378	226		VAC	2.7e-158	208/211	98		(Goebel *et al.*, 1990)
		238		CPX G1L	7.0e-148	166/189	87		(Safronov *et al.*, 1996)
C5L		251		VAR-1	6.6e-147	184/200	92		(Shchelkunov *et al.*, 1995)
030L	26501 26058	147	16.2	dUTPase					(Roseman and Slabaugh, 1990)
F2L		147		VAC	2.9e-102	147/147	100		(Roseman *et al.*, 1996)
		147		CPX G2L	8.2e-100	144/147	97		(Goebel *et al.*, 1990)
C6L		147		VAR	1.1e-97	142/147	96		(Safronov *et al.*, 1996)
		164		human dUTPase	4.1e-61	49/69	71		(Shchelkunov *et al.*, 1995) (Ladner *et al.*, 1996)

TABLE 1—*Continued*

	ORF[a] left	START / STOP terminal	AA[b] region:	kDa[c]	name / (putative) function / homologies[d]	BLAST[e] expect	BLAST[f] AA id	HSS[g] (%)	references
			142		Swinepox virus	8.0e-56	43/70	61	(Massung *et al.*, 1993)
			159		orf virus	1.5e-49	45/69	65	(Mercer *et al.*, 1989)
			178		avian adenovirus	6.6e-49	40/70	57	(Akopian *et al.*, 1992)
			1124		FIV pol polyprotein	1.5e-26	49/117	41	(Talbott *et al.*, 1989)
					dUTPase pyrophosphatase family	>4.2e-06			
X	**031L**	27955 / 26525	476	55.3	**kelch-like protein**				(Senkevich *et al.*, 1993b) (Roseman and Slabaugh, 1990)
	F3L		480		VAC	0.0	292/294	99	(Goebel *et al.*, 1990)
			485		CPX G3L	0.0	287/293	97	(Safronov *et al.*, 1996)
	C7L		179		VAR-1	1.9e-124	166/179	92	(Shchelkunov *et al.*, 1995)
			500		Swinepox virus protein C13	4.4e-46	39/133	29	(Massung *et al.*, 1993)
			564		VAC A55R	2.8e-21	17/51	33	(Goebel *et al.*, 1990)
			689		kelch protein D.melanogaster	5.3e-18	21/65	32	(Xue and Cooley, 1993)
			512		CPX D18L	1.4e-16	15/33	45	(Safronov *et al.*, 1996)
			512		VAC C2L	1.6e-16	15/33	45	(Goebel *et al.*, 1990)
			625		T27E9.4 C. elegans	3.7e-14	15/59	25	Z82059
			624		human KIAA0132 protein	1.9e-13	13/60	21	D50922 o.k
			817		R09A8.3 (C. elegans)	1.1e-12	17/45	37	(Wilson *et al.*, 1994)
			611		C47D12.7 (C. elegans)	2.4e-12	22/91	24	(Wilson *et al.*, 1994)
			530		Swinepox virus	3.0e-09	14/58	24	(Massung *et al.*, 1993)
			589		M M= actin binding protein	1.9e-09	18/88	20	U65079
			521		CPX C3L	1.2e-08	15/37	40	(Safronov *et al.*, 1996)
			509		Myxoma virus MT-9	2.5e-08	17/58	29	(Upton *et al.*, 1990a)
			202		Murine IAP-promoted placenta (MIPP) expressed protein	4.3e-08	17/56	30	(Chang-Yeh *et al.*, 1991)
			326			3.9e-06	22/80	27	Z99708
			559		A. thaliana hyp. protein	9.0e-6	12/31	38	(Senkevich *et al.*, 1993b)
			916		Ectromelia virus p65	0.00016	13/42	30	(Way *et al.*, 1995)
			172		ß-scruin (L. polyphemus)	0.018	15/36	41	(Shchelkunov *et al.*, 1995)
					VAR-1 J8R (BSH: J6R)				
X	**032L**	28925 / 27966	319	37.0	**ribonucleotide reductase (small subunit)**				(Slabaugh *et al.*, 1988) (Roseman and Slabaugh, 1990)
			319		CPX G4L	2.3e-231	317/319	99	(Safronov *et al.*, 1996)
	F4L		319		VAC	3.5e-231	317/319	99	(Goebel *et al.*, 1990)
	C8L		333		VAR-BSH	4.1e-228	313/319	98	(Shchelkunov *et al.*, 1995)
					ribonucleotide reductase family	>2.2e-10			
	033L	29250 / 28957	97	11.1	**36.5k major membrane protein precursor (f1)**				(Roseman and Slabaugh, 1990)
X	C9L		348		VAR-BSH	1.9e-36	51/53	96	(Shchelkunov *et al.*, 1995)
			323		CPX G5L	2.4e-19	47/77	61	(Safronov *et al.*, 1996)
	F5L		321		VAC	3.3e-19	42/70	60	(Goebel *et al.*, 1990)
			1584		non-receptor tyrosin kinase (Dictyostelium discoideum)	0.00038	15/35	42	(Tan and Spudich, 1990)
	034L	29875 / 29219	218	24.8	**36.5k major membrane protein precursor (f2)**				(Roseman and Slabaugh, 1990)
			323		CPX G5L	8.2e-155	215/217	99	(Safronov *et al.*, 1996)
	F5L		321		VAC	6.4e-155	215/217	99	(Goebel *et al.*, 1990)
	C9L		348		VAR-BSH	6.8e-141	186/210	88	(Shchelkunov *et al.*, 1995)
	035L	30129	74	8.6	**8.6k protein**				
	F6L	29905	74		VAC	5.5e-47	74/74	100	(Roseman and Slabaugh, 1990)
	C10L		72		VAR	2.3e-38	62/70	88	(Goebel *et al.*, 1990) (Shchelkunov *et al.*, 1995)
X	**036L**	30387	80	9.4	**9.4k protein**				
	C11L	30145	79		VAR	2.9e-44	34/43	79	(Roseman and Slabaugh, 1990)
	F7L		92		VAC	1.9e-43	65/65	100	(Shchelkunov *et al.*, 1995) (Goebel *et al.*, 1990)
	037L	30731	65	7.9	**7.9k protein**				
	F8L	30534	65		VAC	5.1e-43	63/65	96	(Roseman and Slabaugh, 1990)
	C12L		65		VAR-1	3.1e-41	61/65	93	(Goebel *et al.*, 1990). (Shchelkunov *et al.*, 1995)
	038L	31429	212	23.8	**23.8k protein**				
	F9L	30791	212		VAC	7.1e-148	212/212	100	(Roseman and Slabaugh, 1990)
	C13L		212		VAR	1.2e-144	207/212	97	(Goebel *et al.*, 1990). (Shchelkunov *et al.*, 1995)
			215		Swinepox virus	8.1e-72	39/93	41	(Massung *et al.*, 1993)
	MC016L		213		MCV subtype 1	2.8e-62	71/152	46	(Senkevich *et al.*, 1996)
			225		Orf virus	5.1e-39	27/84	32	(Mercer *et al.*, 1995)
			243		FPV protein FP2	2.8e-17	26/58	44	(Binns *et al.*, 1988)
			243		MCV subtype 1 MC069R	7.7e-12	23/58	39	(Senkevich *et al.*, 1996)
			250		VAC L1R	1.1e-07	20/58	34	(Goebel *et al.*, 1990).
			250		VAR M1R	1.1e-07	20/58	34	(Shchelkunov *et al.*, 1995)
	039L	32735 / 31416	439	52.1	**serine/threonine protein kinase 2**				(Lin and Broyles, 1994)
	F10L		439		VAC	0.0	429/439	97	(Wang and Shuman, 1995)
	C14L		439		VAR-BSH	0.0	424/439	96	(Goebel *et al.*, 1990).
			440		Swinepox virus	2.2e-233	151/214	70	(Shchelkunov *et al.*, 1995)
	MC017L		443		MCV subtype 1	2.3e-198	178/282	63	(Massung *et al.*, 1993)
			498		orf virus	2.2e-162	198/366	54	(Senkevich *et al.*, 1996) (Mercer *et al.*, 1995)
	040L	33012	84	9.6	**39.7k protein (f1)**				
	C15L	32758	354		VAR	6.6e-27	50/64	78	(Shchelkunov *et al.*, 1995)
	F11L		354		VAC	9.1e-27	50/64	78	(Goebel *et al.*, 1990)
	041L	33771	100	11.4	**39.7k protein (f2)**				

TABLE 1—*Continued*

ORF: left	START / STOP terminal	AA: region:	kDa	name / putative function / homologies*	BLAST: expect	BLAST: AA id	BSS (%)	references
F11L	33469	354		VAC	3.8e-62	95/95	100	(Goebel *et al.*, 1990).
C15L		354		VAR	8.8e-58	90/95	94	(Shchelkunov *et al.*, 1995)
042L	35721	635	73.1	73.1k protein				
F12L	33814	635		VAC				
C16L		635		VAR-1	0.0	629/635	99	(Goebel *et al.*, 1990).
		352		Myxoma virus	0.0	607/635	95	(Shchelkunov *et al.*, 1995)
MC019L		663		MCV subtype 1	3.6e-84	28/66	42	U43549
		640		orf virus	4.0e-60	29/82	35	(Senkevich *et al.*, 1996)
		630		FPV F12 homolog	4.8e-39	19/61	31	U34774
					2.3e-15	19/67	28	(Ogawa *et al.*, 1993)
043L	36866 / 35748	372	41.8	37k major EEV antigen IMCBH sensitive protein palmitylprotein				(Hirt *et al.*, 1986) (Schmutz *et al.*, 1991) (Grosenbach *et al.*, 1997)
F13L		372		VAC				
C17L		372		VAR-BSH	2.1e-268	369/372	99	(Goebel *et al.*, 1990)
		371		Myxoma virus	8.9e-265	364/372	97	(Shchelkunov *et al.*, 1995)
		378		orf virus	2.5e-115	110/200	55	U43549
MC021L		388		MCV subtype 1	7.6e-108	83/194	42	(Sullivan *et al.*, 1994)
		377		FPV major env protein	6.1e-98	44/113	38	(Senkevich *et al.*, 1996)
		251		pigeonpox virus	2.8e-88	47/112	41	(Calvert *et al.*, 1992)
		424		CPX M4L	1.8e-62	47/112	41	S27933
		424		VAC K4L	2.1e-18	16/52	30	(Safronov *et al.*, 1996)
		372		D. discoideum	1.7e-17	14/35	40	(Goebel *et al.*, 1990)
		437		HU-K4 (homo sapiens)	1.4e-16	28/84	33	(Giorda *et al.*, 1989)
					1.5e-11	25/94	26	U60644
044L	37105	73	8.3	8.3k protein				
F14L	36884	73		VAC				
C18L		73		VAR	2.3e-44	72/73	98	(Goebel *et al.*, 1990)
					2.1e-35	57/73	78	(Shchelkunov *et al.*, 1995)
045L	378533	158	18.6	18.6k protein				
F15L	37377	158		VAC				
C19L		161		VAR	2.3e-112	157/158	99	(Goebel *et al.*, 1990).
MC025L		148		MCV subtype 1	1.4e-107	150/153	98	(Shchelkunov *et al.*, 1995)
		148		Myxoma virus	3.5e-54	52/113	46	(Senkevich *et al.*, 1996)
					5.4e-50	48/112	42	U43549
046L	38555	231	26.5	26.5k protein				
F16L	37860	231		VAC				
C20L		231		VAR	3.3e-159	227/231	98	(Goebel *et al.*, 1990).
		209		Myxoma virus	5.6e-157	222/231	96	(Shchelkunov *et al.*, 1995)
MC029L		230		MCV subtype 1	8.3e-48	26/58	44	U43549
					6.9e-45	16/61	26	(Senkevich *et al.*, 1996)
047R	38619 / 38924	101	11.3	11k DNA binding phosphoprotein				(Bertholet *et al.*, 1985) (Kao and Bauer, 1987)
F17R		101		VAC				
C21R		101		VAR	3.0e-69	100/101	99	(Goebel *et al.*, 1990)
		102		MYX	9.7e-67	99/101	98	(Shchelkunov *et al.*, 1995)
MC030R		92		MCV subtype 1	6.6e-26	45/92	98	U43549
		46		orf virus	1.5e-20	33/53	48	(Senkevich *et al.*, 1997)
					1.3e-06	16/29	62	(Mercer *et al.*, 1995)
048L	40360 / 38921	479	55.6	poly(A) polymerase catalytic subunit				(Gershon *et al.*, 1991)
E1L		479		VAC				
E1L		479		VAR-1	0.0	478/479	99	(Goebel *et al.*, 1990).
MC031L		470		MCV subtype 1	0.0	472/479	98	(Shchelkunov *et al.*, 1995)
					1.5e-177	114/173	65	(Senkevich *et al.*, 1997)
049L	42570	737	85.9	85.9k protein				
E2L	40357	737		VAC				(Ahn *et al.*, 1990a)
E2L		737		VAR-1	0.0	735/737	99	(Goebel *et al.*, 1990).
MC032L		748		MCV subtype 1	0.0	731/737	99	(Shchelkunov *et al.*, 1995)
					8.3e-127	59/198	29	(Senkevich *et al.*, 1997)
050L	43269 / 42697	190	21.5	dsRNA dependent PK inhibitor, host range				(Chang *et al.*, 1992)
E3L		190		VAC				(Chang *et al.*, 1995b)
E3L		192		VAR-BSH	1.4e-129	188/190	98	(Goebel *et al.*, 1990).
		1175		dsRNA specific ADA (rat)	8.6e-126	111/114	97	(Shchelkunov *et al.*, 1995)
		1226		dsRNA specific ADA (human)	7.2e-12	22/47	46	(O'Connell *et al.*, 1995)
		551		human protein kinase p68	2.8e-09	21/47	44	(Kim *et al.*, 1994)
				INF inducible kinase family	3.8e-05	22/42	52	(Meurs *et al.*, 1990)
					>0.00099			
051L	44103 / 43324	259	29.8	RNA polymerase subunit rpo30, VITF-1				(Ahn *et al.*, 1990a)
E4L		259		VAC				(Broyles and Pennington, 1990)
E4L		259		VAR-BSH	1.6e-182	258/259	99	(Goebel *et al.*, 1990)
MC034L		444		MCV subtype 1	3.2e-180	255/259	98	(Shchelkunov *et al.*, 1995)
		39		orf virus	1.2e-84	107/171	62	(Senkevich *et al.*, 1996)
		243		African swine fever virus	6.7e-10	21/39	53	(Mercer *et al.*, 1995)
				TFIIS family	0.00034	17/36	47	(Vydelingum *et al.*, 1993)
					<0.0096			
052R	44180	331	39.1	39.1k protein				
E5R	45175	331		VAC				(Goebel *et al.*, 1990)
E5R		341		VAR	1.2e-235	329/331	99	(Goebel *et al.*, 1990)
		332		Taterapox	3.1e-223	312/331	94	(Shchelkunov *et al.*, 1995)
		329		Camelpox	7.1e-225	300/314	95	(Douglas and Dumbell, 1996)
		319		Cowpox	1.4e-221	206/220	93	(Douglas and Dumbell, 1996)
		256		Ectromelia	1.5e-202	271/303	89	(Douglas and Dumbell, 1996)
MC038R		276		MCV subtype 1	3.8e-153	218/245	88	(Douglas and Dumbell, 1996)
					8.3e-109	94/152	61	(Senkevich *et al.*, 1997)
053R	45312	567	66.7	66.7k protein				(Goebel *et al.*, 1990)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[b] region:	kDa[c]	name / (putative) function / homologies[d]	BLAST[d] expect	BLAST[e] AA id	HSS[f] (%)	references
E6R		567		VAR	0.0	555/567	97	(Shchelkunov *et al.*, 1995)
MC037R		565		MCV subtype 1	7.2e-247	258/451	57	(Senkevich *et al.*, 1997)
054R	47082	166	19.5	17k myristylprotein				
E7R	47582	166		VAC				(Martin *et al.*, 1997)
E7R		60		VAR-1 (BSH: E6.5R)	9.7e-116	166/166	100	(Goebel *et al.*, 1990)
					2.7e-36	53/60	88	(Shchelkunov *et al.*, 1995)
055R	47695	273	31.9	31.9k protein				
E8R	48516	273		VAC				(Earl *et al.*, 1986)
E8R		273		VAR	4.5e-195	272/273	99	(Goebel *et al.*, 1990)
MC038R		276		MCV subtype 1	9.9e-192	266/273	99	(Shchelkunov *et al.*, 1993a)
					8.3e-109	94/152	97	(Senkevich *et al.*, 1997)
056L	51543	1006	116.9	DNA polymerase				
E9L	48523	1006		VAC				(Earl *et al.*, 1986)
E9L		1005		VAR BSH	0.0	1005/10	99	(Goebel *et al.*, 1990)
		1008		Orf virus	0.0	06	98	(Shchelkunov *et al.*, 1995)
		988		FPV	0.0	598/608	51	(Mercer *et al.*, 1996)
MC039L		1004		MCV subtype 1	0.0	199/388	60	(Binns *et al.*, 1987)
		964		C. biennis poxvirus	0.0	179/294	58	(Senkevich *et al.*, 1997)
				DNA polymerase family	2.6e-77	175/297	34	(Mustafa and Yuen, 1991)
					>6.0e-06	28/82		
057R	51575	95	10.9	10.9k protein				
E10R	51862	95		VAC				(Goebel *et al.*, 1990)
E10R		95		VAR	1.2e-65	93/95	97	(Goebel *et al.*, 1990)
MC040R		101		MCV subtype 1	3.1e-64	90/95	100	(Shchelkunov *et al.*, 1993a)
					5.2e-44	58/95	94	(Senkevich *et al.*, 1997)
058L	52246	129	14.9	14.9k protein				
E11L	51857	129		VAC				(Goebel *et al.*, 1990)
E11L		129		VAR	3.3e-89	129/129	100	(Goebel *et al.*, 1990)
MC041L		132		MCV subtype 1	4.2e-87	125/129	96	(Shchelkunov *et al.*, 1995)
					1.8e-30	31/96	32	(Senkevich *et al.*, 1997)
059L	52691	152	17.6	77.6k protein (f1)				
O1L	52233	666		VAC				(Goebel *et al.*, 1990)
Q1L		666		VAR-BSH	6.9e-101	151/152	99	(Goebel *et al.*, 1990)
MC042L		783		MCV subtype 1	3.4e-92	137/152	90	(Shchelkunov *et al.*, 1995)
				leu zipper, bipartite nuclear targeting sequence	1.5e-22	39/105	37	(Senkevich *et al.*, 1997)
060L	54189	405	47.4	77.6k protein (f2)				(Goebel *et al.*, 1990)
O1L	52972	666		VAC				(Goebel *et al.*, 1990)
Q1L		666		VAR-1	5.8e-277	399/400	99	(Shchelkunov *et al.*, 1995)
MC042L		783		MCV subtype 1	1.7e-269	383/400	95	(Senkevich *et al.*, 1997)
					2.7e-51	38/104	36	
061L	54555	108	12.4	glutaredoxin 1				(Ahn and Moss, 1992a)
	54229							(Johnson *et al.*, 1991)
O2L		108		VAC	2.0e-74	108/108	100	(Goebel *et al.*, 1990)
Q2L		108		VAR	4.9e-72	104/108	96	(Shchelkunov *et al.*, 1995)
		106		human glutaredoxin	3.2e-31	49/106	46	(Fernando *et al.*, 1994)
				glutaredoxin family	>9.0e-05			
062L	55639	312	35.9	35.9k protein				
I1L	54701	312		VAC				(Schmitt and Stunnenberg, 1988)
K1L		312		VAR-BSH	4.7e-208	310/312	99	(Goebel *et al.*, 1990)
MC044L		310		MCV subtype 1	4.8e-205	305/312	97	(Shchelkunov *et al.*, 1995)
		1451		transcription initiation protein (S. cerevisiae)	3.8e-110	163/307	53	(Senkevich *et al.*, 1996)
					0.029	10/28	35	(Hansen *et al.*, 1996)
063L	55867	73	8.5	8.5k protein				
I2L	55646	73		VAC				(Schmitt and Stunnenberg, 1988)
K2L		73		VAR	5.5e-50	73/73	100	(Goebel *et al.*, 1990)
MC045L		72		MCV subtype 1	5.5e-50	73/73	100	(Shchelkunov *et al.*, 1995)
		887		hypothetical yeast protein	3.5e-18	20/33	60	(Senkevich *et al.*, 1996)
					8.1e-05	9/24	37	S48422
064L	56677	269	30.0	DNA binding phospho-protein (F4L interacting)				
	55868							(Schmitt and Stunnenberg, 1988)
I3L		269		VAC				(Davis and Mathews, 1993)
K3L		269		VAR	2.1e-173	267/269	99	(Goebel *et al.*, 1990)
MC046L		288		MCV subtype 1	2.5e-172	265/269	98	(Shchelkunov *et al.*, 1995)
		209		FPV I3 protein	9.6e-66	61/149	40	(Senkevich *et al.*, 1996)
					8.4e-35	23/66	34	A48563
065L	59075	771	87.8	ribonucleotide reductase (large subunit)				
	56760							(Schmitt and Stunnenberg, 1988)
I4L		771		VAC	0.0	771/771	100	(Tengelsen *et al.*, 1988)
K4L		771		VAR	0.0	761/771	98	(Goebel *et al.*, 1990)
				ribonucleotide red family	>1.8e-05			(Shchelkunov *et al.*, 1995)
066L	59342	79	8.8	8.8k protein				
I5L	59103	79		VAC	6.3e-49	79/79	100	(Schmitt and Stunnenberg, 1988)
K5L		79		VAR	1.2e-47	76/79	96	(Goebel *et al.*, 1990)
MC047L		82		MCV subtype 1	2.6e-17	27/73	36	(Shchelkunov *et al.*, 1995)
		81		FPV 9.1K protein	1.4e-12	13/38	34	(Senkevich *et al.*, 1996)
		321		formate dep nitrit reductase protein (H. influenzae)	0.00022	7/18	38	(Binns *et al.*, 1988)
		496		permease (b subtilis)	0.00031	12/43	27	(Fleischmann *et al.*, 1995)
								gi:24153?6
067L	60509	382	43.5	43.5k protein				
I6L	59361	382		VAC	8.6e-268	382/382	100	(Schmitt and Stunnenberg, 1988)
K6L		382		VAR	3.1e-267	380/382	99	(Goebel *et al.*, 1990)
MC048L		406		MCV subtype 1	2.1e-99	44/119	36	(Shchelkunov *et al.*, 1995)
								(Senkevich *et al.*, 1996)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[a] region:	kDa[a]	name / (putative) function / homologies[a]	BLAST[b] expect	BLAST[b] AA id	HSS[c]	references
		390		FPV 16 protein mitochondrial energy transfer proteins signature	1.4e-86	50/136	36	E48563, P12925 (Goebel *et al.*, 1990)
068L	61773 60502	423	49.0	core protein, topoisomerase II				(Schmitt and Stunnenberg, 1988)
I7L		423		VAC				(Kane and Shuman, 1993)
K7L		423		VAR	0.0	420/423	99	(Goebel *et al.*, 1990)
MC049L		515		MCV subtype 1	1.5e-306	419/423	99	(Shchelkunov *et al.*, 1995)
		421		FPV 17 protein	1.9e-199	126/207	60	(Senkevich *et al.*, 1996)
		464		Amsacta moorei poxvirus	8.1e-180	185/340	54	F48563
					3.2e-14	14/47	29	(Hall and Moyer, 1991)
069R	61776 63809	676	77.6	NPH-II, NTPase, RNA helicase				(Shuman, 1992),
I8R		676		VAC				(Koonin and Senkevich, 1992)
K8R		676		VAR	0.0	674/676	99	(Goebel *et al.*, 1990)
MC050R		684		MCV subtype 1	0.0	665/676	98	(Shchelkunov *et al.*, 1995)
		682		FPV virus I8FPV	7.6e-227	144/272	52	(Senkevich *et al.*, 1997)
				61 matches mainly to RNA helicase family	4.2e-206	98/178	55	(Binns *et al.*, 1988)
					<0.38			
070L	65588	591	68.0	68k protein				
G1L	63813	591		VAC				(Schmitt and Stunnenberg, 1988)
H1L		591		VAR-1	0.0	590/591	99	(Goebel *et al.*, 1990)
MC056L		593		MCV subtype 1	0.0	582/591	98	(Shchelkunov *et al.*, 1995)
		341		FPV	1.2e-217	183/361	50	(Senkevich *et al.*, 1997)
					9.4e-75	45/101	44	H48563
071L	65920 65585	111	12.8	12.8k protein				(Schmitt and Stunnenberg, 1988)
G3L		111		VAC				(Meis and Condit, 1991)
H3L		111		VAR	7.6e-74	111/111	100	(Goebel *et al.*, 1990)
MC057L		108		MCV subtype 1	2.4e-71	108/111	97	(Shchelkunov *et al.*, 1995)
					0.00012	15/45	33	(Senkevich *et al.*, 1997)
072R	65914	220	25.8	IBT-dependent protein				(Meis and Condit, 1991)
G2R	66576	220		VAC	1.9e-155	220/220	100	(Goebel *et al.*, 1990)
H2R		220		VAR	1.1e-151	214/220	97	(Shchelkunov *et al.*, 1995)
MC058R		246		MCV subtype 1	2.7e-36	42/135	31	(Senkevich *et al.*, 1997)
073L	66920 66546	124	14.0	glutaredoxin 2 membrane protein				(Gvakharia *et al.*, 1996)
H4L		124		VAR				(Jensen *et al.*, 1996)
G4L		124		VAC	4.0e-83	123/124	99	(Shchelkunov *et al.*, 1995)
MC059L		126		MCV subtype 1	7.5e-83	123/124	99	(Goebel *et al.*, 1990)
					1.1e-21	21/51	41	(Senkevich *et al.*, 1997)
074R	66923	434	49.9	49.8k protein				
G5R	68227	434		VAC				(Goebel *et al.*, 1990)
H5R		434		VAR	1.6e-305	432/434	99	(Goebel *et al.*, 1990)
MC60R		437		MCV subtype 1	1.9e-299	423/434	97	(Shchelkunov *et al.*, 1995)
		1300		HS CG1 protein	1.0e-55	56/119	47	(Senkevich *et al.*, 1997)
					0.015	22/82	26	(Faint *et al.*, 1994)
075R	68235 68426	63	7.3	RNA polymerase subunit rpo7				(Amegadzie *et al.*, 1992),
G5.5R		63		VAC				(Meis and Condit, 1991)
H5.5R		63		VAR	1.1e-40	63/63	100	(Goebel *et al.*, 1990)
MC061R		63		MCV subtype 1	1.1e-39	61/63	96	(Shchelkunov *et al.*, 1995)
				35 matches mainly to RNA polymerases	9.3e-27	41/63	65	(Senkevich *et al.*, 1997)
					<0.54			
076R	68428	165	19.0	18.9k protein				
G6R	68925	165		VAC				(Goebel *et al.*, 1990)
H6R		165		VAR	3.8e-116	162/165	98	(Goebel *et al.*, 1990)
MC062R		195		MCV subtype 1	1.5e-116	164/165	99	(Shchelkunov *et al.*, 1995)
					3.0e-32	27/57	47	(Senkevich *et al.*, 1997)
077L	70005	371	42.0	42.0k protein				
G7L	68890	371		VAC				(Schmitt and Stunnenberg, 1988)
H7L		371		VAR	5.2e-255	370/371	99	(Goebel *et al.*, 1990)
MC065L		402		MCV subtype 1	7.1e-255	369/371	99	(Shchelkunov *et al.*, 1995)
					2.0e-109	69/145	47	(Senkevich *et al.*, 1997)
078R	70036 70818	260	29.9	VLTF-1, late transcription factor				(Keck *et al.*, 1990)
G8R		260		VAC				(Wright *et al.*, 1991)
H8R		260		VAR-1	8.6-184	259/260	99	(Goebel *et al.*, 1990)
MC067R		260		MCV subtype 1	3.1e-183	258/260	99	(Shchelkunov *et al.*, 1995)
		260		FPV virus FP0	8.5e-136	185/260	71	(Senkevich *et al.*, 1997)
					3.3e-129	175/250	67	(Binns *et al.*, 1988)
079R	70838	340	38.9	37k myristylprotein				(Martin *et al.*, 1997)
G9R	71860	340		VAC	3.7e-237	317/319	99	(Goebel *et al.*, 1990)
H9R		340		VAR	9.1e-236	315/319	98	(Shchelkunov *et al.*, 1995)
MC068R		342		MCV subtype 1	4.8e-79	59/127	46	(Senkevich *et al.*, 1997)
		336		FPV virus FP1	3.9e-65	59/124	47	(Binns *et al.*, 1988)
080R	71861 72613	250	27.3	25k myristylprotein IMV virion protein				(Franke *et al.*, 1990)
L1R		250		VAC				(Martin *et al.*, 1997)
M1R		250		VAR	1.8e-175	250/250	100	(Goebel *et al.*, 1990)
MC069R		243		MCV subtype 1	6.4e-170	249/250	99	(Shchelkunov *et al.*, 1995)
		243		FPV virus FP2	6.5e-103	145/243	59	(Senkevich *et al.*, 1997)
		212		VAC F9L	6.2e-95	128/243	52	(Binns *et al.*, 1988)
		212		VAR C13L	1.6e-0.7	20/58	34	(Goebel *et al.*, 1990)
					3.1e-0.7	20/58	34	(Shchelkunov *et al.*, 1995)

TABLE 1—*Continued*

ORF[a]	START / STOP	AA[b]	kDa[c]	name / (putative) function / homologies[d]	BLAST[e] expect	BLAST[f] AA id	HSS[g] (%)	references
left	terminal	region:						
		213		MCV subtype I MC016L	1.6e-0.7	13/57	22	(Senkevich *et al.*, 1997)
		215		swinepox	3.3e-0.5	15/51	29	(Massung *et al.*, 1993)
081R	72645	87	10.3	10.3k protein				
L2R	72908	87		VAC				(Plucienniczak *et al.*, 1985)
M2R		87		VAR	3.9e-57	87/87	100	(Goebel *et al.*, 1990)
MC070R		93		MCV subtype I	4.0e-56	85/87	97	(Shchelkunov *et al.*, 1995)
		504		Na+ dependent phosphate transporter C. elegans	0.064	18/80	22	(Senkevich *et al.*, 1997)
		233		ATPase subunit T. cruzi	6.9e-05	10/39	25	(Wilson *et al.*, 1994)
		2336		Ca^{2+} channel rat	0.013	16/44	36	U38184
		2238		Ca^{2+} channel mouse	5.2e+0.2	6/25	24	(Dubel *et al.*, 1992)
		1559		ABC transporter yeast	7.1e+0.2	6/25	24	(Coppola *et al.*, 1994)
					0.40	12/40	30	X97560
082L	73950	350	40.6	40.6k protein				
L3L	72898	350		VAC				(Plucienniczak *et al.*, 1985)
M3L		349		VAR	2.2e-251	346/350	98	(Goebel *et al.*, 1990)
MC072L		310		MCV subtype I	1.5e-241	296/306	96	(Shchelkunov *et al.*, 1995)
		301		FPV F4 protein	1.5e-88	64/136	47	(Senkevich *et al.*, 1997)
					1.1e-80	58/134	43	(Binns *et al.*, 1988)
083R	73975	251	28.5	core protein VP8 DNA/RNA binding protein				(Yang and Bauer, 1988)
	74730							(Baylis and Smith, 1997)
L4R		251		VAC	5.6e-170	251/251	100	(Goebel *et al.*, 1990)
M4R		251		VAR	3.7-169	250/251	99	(Shchelkunov *et al.*, 1995)
MC073R		254		MCV subtype I	1.7e-76	36/59	61	(Senkevich *et al.*, 1997)
		253		FPV virus FP5	6.4e-55	29/57	50	(Binns *et al.*, 1988)
084R	74740	128	15.1	15.1k protein				
L5R	75126	128		VAC 14.0k protein	2.9e-89	127/128	99	(Goebel *et al.*, 1990)
M5R		128		VAR	2.0-87	125/128	97	(Shchelkunov *et al.*, 1995)
		129		FPV FP6	8.1e-16	19/45	42	(Drillien *et al.*, 1987)
MC074R		146		MCV subtype I	0.073	10/18	55	(Senkevich *et al.*, 1997)
		152		melatonin receptor D. rerio	0.44	15/66	222	(Reppert *et al.*, 1995)
085R	75083	153	17.9	dimeric virion protein				
J1R	75544	153		VAC	6.0e-103	152/153	99	(Holzer & Falkner, unpubl.)
L1R		159		VAR-I	1.4e-101	149/153	97	(Goebel *et al.*, 1990)
		147		capripox CF7	6.5e-54	53/90	58	(Shchelkunov *et al.*, 1995)
		148		myxoma MF7	4.8e-51	54/93	58	(Gershon and Black, 1989b)
		183		MCV subtype I	1.9e-47	47/93	50	(Jackson and Bults, 1992)
MC075R		148		FPV FP7	1.3e-35	37/84	44	(Senkevich *et al.*, 1997)
								(Drillien *et al.*, 1987)
086R	75560	177	20.0	thymidine kinase				
	76093							(Hruby and Ball, 1982)
J2R		177		VAC				(Weir and Moss, 1983)
L2R		177		VAR	5.7e-125	175/177	98	(Goebel *et al.*, 1990)
				38 matches mainly to thymidine kinase family	2.7e-122	170/177	96	(Shchelkunov *et al.*, 1995)
					<0.18			
087R	76159	333	38.9	poly(A) polymerase su, 2'methyl transferase				
	77160							(Gershon *et al.*, 1991)
J3R		333		VAC				(Gershon and Moss, 1993)
L3R		333		VAR-BSH	8.7e-136	330/333	99	(Goebel *et al.*, 1990)
		338		myxoma	9.8e-233	326/333	97	(Shchelkunov *et al.*, 1995)
MC076R		343		MCV subtype I	5.7e-288	247/333	74	(Jackson and Bults, 1990)
		308		FPV VP39	1.4e-135	79/144	54	(Senkevich *et al.*, 1997)
					1.7e-96	125/267	46	(Binns *et al.*, 1988)
088R	77075	185	21.3	RNA pol subunit rpo22				
J4R	77632	185		VAC				(Broyles and Moss, 1986)
L4R		185		VAR-BSH	1.2e-125	185/185	100	(Goebel *et al.*, 1990)
		185		myxoma	7.9e-125	182/185	98	(Shchelkunov *et al.*, 1995)
MC077R		187		MCV subtype I	1.5e-86	124/185	67	(Jackson and Bults, 1990)
		186		FPV	1.9e-76	73/132	55	(Senkevich *et al.*, 1997)
					2.1e-73	72/135	53	(Binns *et al.*, 1988)
089L	78101	133	15.2	15.2k protein				
J5L	77700	133		VAC				(Plucienniczak *et al.*, 1985)
L5L		133		VAR-I	2.4e-95	133/133	100	(Goebel *et al.*, 1990)
MC078L		134		MCV subtype I	2.4e-94	131/133	98	(Shchelkunov *et al.*, 1995)
		137		FPV	5.7e-45	60/127	47	(Senkevich *et al.*, 1997)
		377		VAR-I A16L (BSH:A17L)	1.4e-43	60/130	46	(Drillien *et al.*, 1987)
		378		VAC A16L	0.049	7/28	25	(Shchelkunov *et al.*, 1995)
					0.049	7/28	25	(Goebel *et al.*, 1990)
090R	78207	1286	146.9	RNA pol subunit rpo147				
J6R	82067	1286		VAC				(Broyles and Moss, 1986)
L6R		1286		VAR	0.0	1283/1286	99	(Goebel *et al.*, 1990)
MC079R		1289		MCV subtype I	0.0	1275/1286	99	(Shchelkunov *et al.*, 1995)
				100 matches to RNA pol (large subunit) family	0.0	556/760	73	(Senkevich *et al.*, 1997)
					<3.7e-07			
091L	82579	171	19.7	protein tyrosine/serine phosphatase				
	82064							(Rosel *et al.*, 1986)
H1L		171		VAC				(Guan *et al.*, 1991)
H1L		171		VAR	2.0e-117	170/171	99	(Goebel *et al.*, 1990)
		171		raccoonpox	1.1e-114	166/171	97	(Shchelkunov *et al.*, 1995)
		172		myxoma virus	6.0e-111	157/171	91	B47452
		173		rabbit fibroma virus	1.5e-77	83/138	60	(Mossman *et al.*, 1995a)
MC082L		169		MCV subtype I	1.8e-77	46/80	57	(Mossman *et al.*, 1995a)
				protein phosphatase family	1.4e-65	60/114	52	(Senkevich *et al.*, 1997)
					>2.8e-05			

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[a] region	kDa[a]	name / (putative) function / homologies[a]	BLAST[c] expect	BLAST[c] AA id	HSS (%)	references
092R	82593	189	21.5	21.5k protein				
H2R	83162	189		VAC				(Rosel *et al.*, 1986)
I2R		189		VAR	5.2e-134	188/189	99	(Goebel *et al.*, 1990)
MC083R		191		MCV subtype I	1.4e-133	188/189	99	(Shchelkunov *et al.*, 1995)
		142		myxoma	1.4e-71	95/181	52	(Senkevich *et al.*, 1997)
					1.3e-65	93/142	65	(Jackson and Bults, 1990)
093L	84139 / 83165	324	37.5	immunodominant env protein p35; IMV membrane-associated				(Rosel *et al.*, 1986) (Chertov *et al.*, 1991) (Takahashi *et al.*, 1994)
H3L		324		VAC				
I3L		325		VAR-BSH	3.3e-231	322/324	99	(Goebel *et al.*, 1990)
MC084L		298		MCV subtype I	1.7e-225	311/320	97	(Shchelkunov *et al.*, 1995)
					1.1e-36	38/117	32	(Senkevich *et al.*, 1996)
094L	86527 / 84140	795	93.6	RAP 94 (RNA-pol assoc. transcr. spec. factor)				(Ahn and Moss, 1992b) (Kane and Shuman, 1992)
H4L		795		VAC				
I4L		795		VAR	0.0	791/795	99	(Goebel *et al.*, 1990)
MC085L		791		MCV subtype I	0.0	780/795	98	(Shchelkunov *et al.*, 1995)
		804		Orf virus	0.0	327/546	59	(Senkevich *et al.*, 1996)
		484		FPV L1L protein	0.0	96/131	73	(Fleming *et al.*, 1993)
					2.4e-181	91/176	51	2209386A
095R	86713 / 87324	203	22.3	late transcription factor VLTF-4				(Kovacs and Moss, 1996)
H5R		203		VAC				(Rosel *et al.*, 1986)
I5R		221		VAR	1.8e-128	202/203	99	(Goebel *et al.*, 1990)
		227		orf virus F3R	5.1e-102	91/97	93	(Shchelkunov *et al.*, 1995)
		220		MCV subtype I	3.1e-14	29/69	42	(Fleming *et al.*, 1993)
		705		nucleolin Xenopus	3.1e-09	28/64	43	(Senkevich *et al.*, 1997)
				31 matches to glu/asp rich proteins	0.00041 E<0.52	18/57	31	(Messmer and Dreyer, 1993)
096R	87325 / 88269	314	36.7	DNA topoisomerase I				(Shuman and Moss, 1987)
H6R		314		VAC				(Rosel *et al.*, 1986)
I6R		314		VAR-BSH	0.0	314/314	100	(Goebel *et al.*, 1990)
		314		shope fibroma virus	9.5e-220	312/314	99	(Shchelkunov *et al.*, 1995)
		318		orf virus	8.5e-141	119/170	70	(Upton *et al.*, 1990b)
MC087R		323		MCV subtype I	5.2e-128	82/138	59	(Fleming *et al.*, 1993)
		316		FPV L3R	1.6e-121	111/202	54	(Senkevich *et al.*, 1997)
				21 matches to topoisomerase family	2.9e-113	159/303	52	(Zantinge *et al.*, 1996)
097R	88306 / 88746	146	17.0	17.0k protein				(Rosel *et al.*, 1986)
H7R		146		VAC				(Goebel *et al.*, 1990)
I7R		146		VAR	2.1e-98	144/146	98	(Shchelkunov *et al.*, 1995)
MC088R		143		MCV subtype I	6.7e-96	141/146	96	(Senkevich *et al.*, 1997)
					4.3e-30	45/115	39	
098R	88790 / 91324	844	96.8	mRNA capping enzyme, large subunit				(Morgan *et al.*, 1984)
D1R		844		VAC				(Niles *et al.*, 1986)
F1R		844		VAR-BSH	0.0	842/844	99	(Goebel *et al.*, 1990)
MC090R		950		MCV subtype I	0.0	830/844	98	(Shchelkunov *et al.*, 1995)
		836		shope fibroma virus	0.0	322/64	64	(Senkevich *et al.*, 1997)
		868		ASV NP868R	0.0	243/305	79	(Upton *et al.*, 1991b)
					0.0033	17/55	30	(Pena *et al.*, 1993)
099L	91723 / 91283	146	16.9	structural protein				(Niles *et al.*, 1986)
D2L		146		VAC				(Dyster and Niles, 1991)
F2L		146		VAR (BSH: F3L)	5.9e-98	146/146	100	(Goebel *et al.*, 1990)
		143		Rabbit fibroma virus	1.5e-97	145/146	99	(Shchelkunov *et al.*, 1995)
MC091L		170		MCV subtype I	2.0e-27	13/33	39	(Upton *et al.*, 1991b)
					1.1e-20	19/41	46	(Senkevich *et al.*, 1996)
100R	91716	233	27.6	27k structural protein				(Dyster and Niles, 1991)
D3R	92417	237		VAC	3.8-167	136/142	95	(Goebel *et al.*, 1990)
F2R		237		VAR I:F3R	1.5e-162	131/142	92	(Shchelkunov *et al.*, 1995)
		241		shope fibroma virus	9.3e-20	27/100	27	(Upton *et al.*, 1991b)
MC092R		268		MCV subtype I	3.5e-18	16/39	41	(Senkevich *et al.*, 1997)
		206		rabbit fibroma virus C3	1.6e-09	26/96	27	(Strayer *et al.*, 1991)
101R	92417	218	25.1	uracil DNA glycosylase				(Upton *et al.*, 1993)
D4R	93073	218		VAC	1.4e-157	217/218	99	(Goebel *et al.*, 1990)
F4R		218		VAR-BSH	5.1e-157	216/218	99	(Shchelkunov *et al.*, 1995)
		218		shope fibroma virus	1.5e-117	151/218	69	(Upton *et al.*, 1993)
MC093R		226		MCV subtype I	8.4e-91	65/113	57	(Senkevich *et al.*, 1997)
		218		FPV FPD4	3.1e-88	116/216	53	(Tartaglia *et al.*, 1990)
		297		uracil DNA glycosylase UL2 gallid herpesvirus 1	0.019	8/14	57	L34064
102R	93105 / 95462	785	90.4	90.4k ATP/GTP binding protein				(Niles *et al.*, 1986)
D5R		785		VAC				(Shchelkunov *et al.*, 1993c)
F5R		785		VAR	0.0	780/785	99	(Goebel *et al.*, 1990)
		786		shope fibroma C5	0.0	774/785	98	(Shchelkunov *et al.*, 1995)
		791		MCV subtype I	0.0	283/450	62	(Strayer *et al.*, 1991)
		791		FPV virus FPD5	0.0	184/334	55	(Senkevich *et al.*, 1997)
MC094R		942		C29E6.4 C. elegans	0.0	170/345	49	(Tartaglia *et al.*, 1990)
					0.72	16/56	28	(Wilson *et al.*, 1994)
103R	95503 / 97416	637	73.9	early transcription factor VETF-1				(Broyles and Fesler, 1990) (Gershon and Moss, 1990)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[b] region:	kDa[b]	name / (putative) function / homologies[c]	BLAST[d] expect	BLAST[e] AA id	HSS[f] (%)	references
D6R		637		VAC	0.0	635/637	99	(Goebel *et al.*, 1990)
F6R		637		VAR-1	0.0	633/637	99	(Shchelkunov *et al.*, 1995)
		635		shope fibroma virus	0.0	212/262	80	(Strayer *et al.*, 1991)
MC095R		635		MCV subtype 1	0.0	199/263	75	(Senkevich *et al.*, 1997)
		605		FPV	0.0	188/263	71	(Binns *et al.*, 1990) (Tartaglia *et al.*, 1990)
		648		Choristoneura biennis EPV	2.7e-08	24/72	33	(Yuen *et al.*, 1991)
		648		Amsacta moorei EPV	4.2e-06	24/77	31	(Hall and Moyer, 1991)
		706		African swine fever virus	1.5e-05	13/38	34	(Yanez *et al.*, 1993)
104R	97443 97928	161	17.9	**RNA polymerase subunit rpo18**				(Ahn *et al.*, 1990b) (Quick and Broyles, 1990)
D7R		161		VAC	1.4e-108	160/161	99	(Goebel *et al.*, 1990)
F7R		161		VAR	2.2e-106	156/161	96	(Shchelkunov *et al.*, 1995)
		163		rabbit fibroma C8	3.4e-76	108/161	67	(Strayer *et al.*, 1991)
MC097R		161		MCV subtype 1	4.0e-70	99/158	62	(Senkevich *et al.*, 1997)
		161		FPV D7	5.4e-66	95/160	59	(Binns *et al.*, 1990)
105L	98805 97891	304	35.4	**virion transmembrane protein, carbonic anhydrase-like**				(Niles and Seto, 1988) (Niles *et al.*, 1986) (Maa *et al.*, 1990)
D8L		304		VAC	2.3e-212	297/304	97	(Goebel *et al.*, 1990)
F8L		304		VAR	2.5e-209	291/304	95	(Shchelkunov *et al.*, 1995)
		304		Camelpox virus	1.1e-207	290/304	95	X97857
		303		Ectromelia virus	2.2e-207	195/207	94	X97856
		304		Monkeypox virus	3.0e-207	287/304	94	X97855
		304		Cowpox virus	9.8e-206	285/304	93	X97858
				Carbonic anhydrase family	>4.9e-13			
106R	98847 99488	213	25.0	**25k mutT-like protein**				(Koonin, 1993) (Niles *et al.*, 1986)
D9R		213		VAC	1.6e-146	212/213	99	(Goebel *et al.*, 1990)
F9R		213		VAR	5.3e-145	209/213	98	(Shchelkunov *et al.*, 1995)
		218		rabbit fibroma	1.7e-75	105/203	51	(Strayer *et al.*, 1991)
MC098R		212		MCV subtype 1	5.3e-67	54/111	48	(Senkevich *et al.*, 1997)
		78		FPV D9	2.0e-13	25/51	49	(Tartaglia *et al.*, 1990)
MC099R		229		MCV subtype 1	0.0041	13/31	41	(Senkevich *et al.*, 1997)
		248		VAR-1 F10R	0.018	14/32	43	(Shchelkunov *et al.*, 1995)
		225		FPV D10	0.14	15/34	44	(Tartaglia *et al.*, 1990)
		248		VAC D10R	0.23	11/26	42	(Goebel *et al.*, 1990)
107R	99485 100231	248	28.9	**29k mutT-like protein**				(Koonin, 1993) (Niles *et al.*, 1986)
D10R		248		VAC	7.4e-173	245/248	98	(Goebel *et al.*, 1990)
F10R		248		VAR-1	5.4e-173	245/248	98	(Shchelkunov *et al.*, 1995)
		260		shope fibroma D10	3.8e-72	96/202	47	(Strayer *et al.*, 1991)
MC099R		229		MCV subtype 1	1.4e-54	44/100	44	(Senkevich *et al.*, 1997)
		225		FPV D10	1.1e-45	45/102	44	(Binns *et al.*, 1990)
		218		shope fibroma D9	1.9e-06	19/54	35	(Strayer *et al.*, 1991)
		212		MCV subtype 1 MC098R	0.13	12/21	57	(Senkevich *et al.*, 1997)
		136		mutator Synechocystis	0.23	12/27	44	D90899
		213		VAC D9R	0.24	11/26	42	(Goebel *et al.*, 1990)
		213		VAR F9R	0.24	11/26	42	(Shchelkunov *et al.*, 1995)
		169		mutator M. jannaschii	0.39	13/25	52	(Bult *et al.*, 1996)
108L	102127 100232	631	72.4	**nucleoside triphosphate phosphohydrolase I, DNA helicase**				(Broyles and Moss, 1987) (Rodriguez *et al.*, 1986) (Koonin and Senkevich, 1992)
D11L		631		VAC	0.0	629/631	99	(Goebel *et al.*, 1990)
N1L		631		VAR	0.0	626/631	99	(Shchelkunov *et al.*, 1995)
MC100R		634		MCV subtype 1	7.3e-286	392/627	62	(Senkevich *et al.*, 1996)
		637		FPV protein 5	2.8e-275	214/357	59	S42251
		370		Rabbit fibroma C14 protein	1.8e-176	244/368	66	F36819
		648		AmEPV	6.0e-142	81/159	50	(Hall and Moyer, 1991)
		648		Choristoneura biennis EPV	1.1e-136	81/158	51	(Yuen *et al.*, 1991)
		89		Swinepox virus	1.2e-34	60/89	67	(Massung *et al.*, 1993)
		1098		ASF	1.6e-13	26/89	29	(Baylis *et al.*, 1993)
		1085		RAD26 (yeast)	5.1e-05	16/45	35	(Huang *et al.*, 1994)
		769		HS transcription activator NTPase family	0.00093 >5.1e-5	10/22	45	(Okabe *et al.*, 1992)
109L	103025 102162	287	33.3	**mRNA capping enzyme, transcription initiation factor VITF**				(Niles *et al.*, 1989) (Weinrich and Hruby, 1986) (Vos *et al.*, 1991)
D12L		287		VAC	2.0e-198	285/287	99	(Goebel *et al.*, 1990)
N2L		287		VAR	9.8e-198	284/287	99	(Shchelkunov *et al.*, 1995)
		287		Swinepox virus	4.1e-160	220/287	76	(Massung *et al.*, 1993)
MC101L		295		MCV subtype 1	5.8e-126	171/279	61	(Senkevich *et al.*, 1996)
		289		FPV protein 6	3.4e-113	114/215	53	S42252
110L	104711 103056	551	61.9	**rifampicin resistance gene, IMV protein**				(Tartaglia and Paoletti, 1985) (Weinrich and Hruby, 1986)
D13L		551		VAC	0.0	551/551	100	(Goebel *et al.*, 1990)
N3L		551		VAR	0.0	547/551	99	(Shchelkunov *et al.*, 1995)
		551		Swinepox virus	4.5e-286	357/506	70	(Massung *et al.*, 1993)
MC102L		547		MCV subtype 1	5.4e-248	298/494	60	(Senkevich *et al.*, 1996)
		552		FPV protein 7	6.6e-223	182/305	59	S42253
		584		Heliothis armigera EPV	9.5e-51	54/107	50	(Osborne *et al.*, 1996)

TABLE 1—*Continued*

ORF left	ORF terminal	START / STOP region	AA	kDa	name / (putative) function / homologies	BLAST expect	BLAST AA id	BLAST %	HSS	references
111L	105187	150	16.8	late gene trans-activator, VLTF-2						(Weinrich and Hruby, 1986)
	104735									(Keck *et al.*, 1993)
A1L		150		VAC		6.8e-103	149/150	99		(Goebel *et al.*, 1990)
A1L		150		VAR		6.8e-103	149/150	99		(Shchelkunov *et al.*, 1995)
MC103L		169		MCV subtype 1		6.3e-54	74/147	50		(Senkevich *et al.*, 1996)
		154		FPV protein 8		2.8e-50	50/87	57		S42254
112L	105882	224	26.3	late gene trans-activator						(Weinrich and Hruby, 1986)
A2L	105208			VAC						(Passarelli *et al.*, 1996)
A2L		224		VAR		1.3e-158	224/224	100		(Goebel *et al.*, 1990)
MC104L		224		MCV subtype 1		1.3e-158	224/224	100		(Shchelkunov *et al.*, 1995)
		228		orf virus		6.4e-127	172/222	77		(Senkevich *et al.*, 1996)
		606				6.8e-30	43/66	65		(Mercer *et al.*, 1995)
113L	106109	76	8.9	8.9k protein						
	105879	76		VAC-WR		1.6e-47	73/76	96		(Weinrich and Hruby, 1986)
A3L		76		VAR-BSH (I:A2.5L)		2.1e-47	71/76	93		(Shchelkunov *et al.*, 1995)
MC105L		70		MCV subtype 1		9.8e-12	26/63	41		(Senkevich *et al.*, 1996)
114L	108058	644	72.6	major core protein P4b						(Rosel and Moss, 1985)
A3L	106124	644		VAC		0.0	643/644	99		(Goebel *et al.*, 1990)
A4L		644		VAR-BSH (I:A3L)		0.0	636/644	98		(Shchelkunov *et al.*, 1995)
MC106L		675		MCV subtype 1		8.9e-272	227/357	63		(Senkevich *et al.*, 1996)
		657		FPV Major core protein P4b		9.1e-220	169/299	56		(Binns *et al.*, 1989)
115L	108929	272	29.9	membrane associated core protein						(Demkowicz *et al.*, 1992)
	108111									(Cudmore *et al.*, 1996)
A4L		281		VAC		1.1e-145	180/187	96		(Goebel *et al.*, 1990)
A5L		271		VAR-BSH (I: A4L)		1.1e-112	165/178	92		(Shchelkunov *et al.*, 1995)
		268		Thermoproteus phage 1		1.9e-09	38/127	29		(Neumann and Zillig, 1990)
		5179		human mucin		4.5e-07	34/139	24		(Gum *et al.*, 1994)
				many matches to Pro-rich proteins						
116R	108967	164	19.0	RNA pol subunit rpo19						(Ahn *et al.*, 1992)
A5R	109461	164		VAC		5.8e-110	164/164	100		(Goebel *et al.*, 1990)
A5R		164		VAR-I (BSH:A6R)		7.0e-109	162/164	98		(Shchelkunov *et al.*, 1995)
MC108R		165		MCV subtype 1		3.3e-51	82/151	53		(Senkevich *et al.*, 1997)
		167		FPV		3.3e-51	72/161	44		(Kumar and Boyle, 1990)
				54 matches/glu-rich proteins		<0.51				
117L	110576	372	43.1	43.1k protein						
A6L	109458	372		VAC		1.2e-248	371/372	99		(Goebel *et al.*, 1990)
A7L		372		VAR-BSH (I: A6L)		1.1e-244	364/372	97		(Shchelkunov *et al.*, 1995)
MC109L		461		MCV subtype 1		4.0e-99	132/367	35		(Senkevich *et al.*, 1996)
		339		FPV ORF 2 protein		1.9e-95	111/279	39		B60013
118L	112732	710	82.3	VETF 82k subunit						(Gershon and Moss, 1990)
A7L	110630	710		VAC		0.0	708/710	99		(Goebel *et al.*, 1990)
A8L		710		VAR-BSH (I: A7L)		0.0	700/710	98		(Shchelkunov *et al.*, 1995)
MC110L		707		MCV subtype 1		0.0	240/374	64		(Senkevich *et al.*, 1996)
119R	112786	288	33.6	33.6k protein VITF-3 subunit						(Van Meir and Wittek, 1988)
A8R	113652	288		VAC		5.3e-198	287/288	99		(Goebel *et al.*, 1990)
A8R		288		VAR-I (BSH:A9R)		3.1e-195	284/288	98		(Shchelkunov *et al.*, 1995)
MC111R		435		MCV subtype 1		4.4e-94	100/169	59		(Senkevich *et al.*, 1997)
120L	113929	94	10.5	10.5k protein						(Van Meir and Wittek, 1988)
A10L	113645	95		VAR-BSH (I: A9L)		9.0e-59	78/79	98		(Shchelkunov *et al.*, 1995)
A9L		99		VAC		9.4e-55	82/91	90		(Goebel *et al.*, 1990)
MC112L		128		MCV subtype 1		1.0e-29	47/71	66		(Senkevich *et al.*, 1996)
		69		orf virus		3.0e-16	27/45	60		(Mercer *et al.*, 1995)
121L	116605	891	102.2	major core protein P4a						(Van Meir and Wittek, 1988)
	113930									(Vansiyke *et al.*, 1991)
A10L		891		VAC		0.0	883/891	99		(Goebel *et al.*, 1990)
A11L		892		VAR-BSH (I: A10L)		0.0	442/463	95		(Shchelkunov *et al.*, 1995)
MC113L		889		MCV subtype 1		5.8e-289	99/177	55		(Senkevich *et al.*, 1996)
122R	116620	318	36.1	36.1k protein						(Goebel *et al.*, 1990)
A11R	117576	318		VAC		3.5e-212	318/318	100		(Goebel *et al.*, 1990)
A11R		319		VAR-I (BSH: A12R)		2.7e-154	242/277	87		(Shchelkunov *et al.*, 1995)
MC114R		304		MCV subtype 1		2.9e-98	92/154	59		(Senkevich *et al.*, 1997)
		148		FPV 4a gene		1.9e-13	18/32	56		A20158
123L	118141	187	20.0	virion protein						(Takahashi *et al.*, 1994)
A12L	117578	192		VAC		4.8e-127	127/128	99		(Goebel *et al.*, 1990)
A13L		189		VAR-BSH (I: A12L)		5.9e-64	101/144	70		(Shchelkunov *et al.*, 1995)
MC115L		178		MCV subtype 1		5.9e-37	39/83	46		(Senkevich *et al.*, 1996)
124L	118377	70	7.6	structural protein						(Takahashi *et al.*, 1994)
	118165			IMV membrane protein						(Jensen *et al.*, 1996)
		70		p8		2.4e-42	66/69	95		(Goebel *et al.*, 1990)
A13L		68		VAC		4.1e-20	37/64	57		(Shchelkunov *et al.*, 1995)
A14L				VAR-BSH (I: A13L)						
125L	118757	90	10.0	structural protein						(Takahashi *et al.*, 1994)
	118485			IMV membrane protein p16						(Jensen *et al.*, 1996)

TABLE 1—*Continued*

ORF[a]	START STOP	AA[b]	kDa[c]	name / (putative) function / homologies[d]	BLAST[e] expect	BLAST[e] AA id	HSS[f] (%)	references
left terminal region:								
A14L		90		VAC	5.3e-62	90/90	100	(Goebel *et al.*, 1990)
A15L		90		VAR-BSH (I: A14L)	5.3e-61	88/90	97	(Shchelkunov *et al.*, 1995)
MC118L		94		MCV subtype I	7.3e-22	31/72	43	(Senkevich *et al.*, 1996)
		125		human interferon inducible protein	0.23	15/49	30	(Deblandre *et al.*, 1995)
126L	119209	94	11.0	11k protein				
A15L	118925	94		VAC	4.1e-63	94/94	100	(Goebel *et al.*, 1990)
A16L		94		VAR-BSH (I:A15L)	1.0e-61	92/94	97	(Shchelkunov *et al.*, 1995)
MC120L		96		MCV subtype I	6.7e-08	17/51	33	(Senkevich *et al.*, 1996)
127L	120326	377	43.4	35k myristylprotein				(Martin *et al.*, 1997)
A16L	119193	378		VAC	6.3e-288	327/327	100	(Goebel *et al.*, 1990)
A17L		377		VAR-BSH (I:A16L)	1.5e-283	368/377	97	(Shchelkunov *et al.*, 1995)
MC121L		364		MCV subtype I	6.5e-110	45/115	39	(Senkevich *et al.*, 1996)
128L	120940 120329	203	23.0	IMV membrane protein morphogenesis factor				(Krijnse-Locker *et al.*, 1996) (Rodriguez *et al.*, 1995) (Wolffe *et al.*, 1996)
A17L		203		VAC	1.0e-141	201/203	99	(Goebel *et al.*, 1990)
A18L		203		VAR-BSH (I:A17L)	1.0e-141	201/203	99	(Shchelkunov *et al.*, 1995)
MC122L		179		MCV subtype I	1.4e-47	36/81	44	(Senkevich *et al.*, 1996)
129R	120955 122436	493	56.8	DNA helicase DNA dependent ATPase				(Koonin and Senkevich, 1992) (Bayliss and Condit, 1995)
A18R		493		VAC	0.0	488/493	98	(Goebel *et al.*, 1990)
A18R		493		VAR-I (BSH:A19R)	0.0	478/493	96	(Shchelkunov *et al.*, 1995)
MC123R		694		MCV subtype I	5.3e-167	203/403	50	(Senkevich *et al.*, 1997)
		450		Bacteriophage T5 D10 helicase-like protein	0.0066	13/36	36	P11107
130L	122650	77	8.3	8.3kb protein				(Goebel *et al.*, 1990)
A19L	122417	77		VAC	2.9e-50	77/77	100	(Goebel *et al.*, 1990)
A19L		76		VAR-I (BSH: A20L)	1.2e-34	54/64	84	(Shchelkunov *et al.*, 1995)
MC124L		78		MCV subtype I	1.5e-13	14/37	37	(Senkevich *et al.*, 1996)
		1721		HS RIZ, zink finger protein	0.0060	7/16	43	(Buyse *et al.*, 1995)
131L	123004	117	13.6	13.6k protein				(Goebel *et al.*, 1990)
A21L	122651	117		VAC	5.3e-83	117/117	100	(Goebel *et al.*, 1990)
A22L		117		VAR-BSH (I: A20L)	7.2e-82	115/117	98	(Shchelkunov *et al.*, 1995)
MC125L		114		MCV subtype I	2.8e-28	23/41	56	(Senkevich *et al.*, 1996)
132R	123003	426	49.1	49.1k protein				(Goebel *et al.*, 1990)
A20R	124283	426		VAC	7.6e-298	423/426	99	(Goebel *et al.*, 1990)
A21R		426		VAR	1.6e-294	418/426	98	(Shchelkunov *et al.*, 1995)
MC126R		476		MCV subtype I	3.2e-95	34/131	25	(Senkevich *et al.*, 1997)
		1118		Pichia klyveri DNA pol	0.069	12/54	22	Y11606
133R	124213	187	21.9	21.9k protein				(Goebel *et al.*, 1990)
A22R	124776	187		VAR-I (BSH:A23R)	1.1e-126	182/187	97	(Shchelkunov *et al.*, 1995)
A22R		176		VAC	1.2e-122	174/176	98	(Goebel *et al.*, 1990)
MC127R		282		MCV subtype I	5.8e-43	35/85	41	(Senkevich *et al.*, 1997)
134R	124796	382	44.6	44.6k protein VITF-3 subunit				(Goebel *et al.*, 1990)
A23R	125944	382		VAC	4.2e-269	382/382	100	(Goebel *et al.*, 1990)
A23R		382		VARI (BSH:A24R)	1.7e-265	377/382	98	(Shchelkunov *et al.*, 1995)
MC128R		383		MCV subtype I	3.5e-136	83/143	58	(Senkevich *et al.*, 1997)
135R	125966 129436	1155	132.4	RNA pol subunit rpo132				(Hooda-Dhingra *et al.*, 1990) (Amegadzie *et al.*, 1991b)
A24R		1164		VAC	0.0	794/796	99	(Goebel *et al.*, 1990)
		1164		CPX rpo132	0.0	794/795	99	(Patel and Pickup, 1989)
A25R		1164		VAR-BSH (I:A24R)	0.0	789/795	99	(Shchelkunov *et al.*, 1995)
MC129R		1165		MCV subtype I	0.0	441/565	78	(Senkevich *et al.*, 1997)
		1162		orf virus	0.0	166/258	64	U33419
				101 matches to RNA pol beta subunit family	<0.036			
right terminal region:								
136L	129638	65	7.5	150k CPX-ATI (f)				(Funahashi *et al.*, 1988)
A25L	129441	65		VAC	1.3e-41	64/65	98	(Goebel *et al.*, 1990)
		1284		Cowpox (CPX-ATI)	3.2e-15	28/30	93	(Funahashi *et al.*, 1988)
137L	130916	230	27.1	27.1k protein (f)				(Amegadzie *et al.*, 1991a)
A30L	130224	498		VAR-BSH (I: A29L)	3.1e-158	216/227	95	(Shchelkunov *et al.*, 1995)
A26L		322		VAC (ATI flanking protein)	5.6e-142	195/197	98	(Goebel *et al.*, 1990)
MC131L		513		MCV subtype I	2.1e-12	19/59	32	(Senkevich *et al.*, 1996)
MC133L		546		MCV subtype I	4.2e-11	12/40	30	(Senkevich *et al.*, 1996)
MC130L		451		MCV subtype I	2.3e-10	14/40	35	(Senkevich *et al.*, 1996)
		702		VAR-I A28L (BSH:A29L)	0.0021	12/37	32	(Shchelkunov *et al.*, 1995)
		726		Camelpox	0.051	11/37	29	(Meyer and Rziha, 1993)
138L	131298 130966	110	12.5	14k membrane protein EEV protein fusion protein				(Rodriguez and Esteban, 1987) (Rodriguez and Smith, 1990) (Gong *et al.*, 1990)
A27L		110		VAC	3.3e-70	108/110	98	(Goebel *et al.*, 1990)
A31L		110		VAR-BSH (I: A30L)	1.1e-69	107/110	97	(Shchelkunov *et al.*, 1995)
		117		Camelpox virus	1.5e-69	106/110	96	(Meyer *et al.*, 1994)
		110		Cowpox virus	1.6e-69	107/110	97	(Meyer *et al.*, 1994)

TABLE 1 — *Continued*

ORF[a]	START / STOP	AA	kDa[b]	name / (putative) function / homologies[c]	BLAST[d] expect	BLAST[e] AA id	HSS[f] (%)	references
left terminal region:								
		110		Ectromelia virus	6.7e-68	105/110	95	(Meyer *et al.*, 1994)
		110		Monkeypox virus	8.3e-67	103/110	93	(Meyer *et al.*, 1994)
		89		Orf virus	4.8e-15	22/57	38	(Naase *et al.*, 1991)
		188		Myxoma virus	2.5e-12	18/33	54	(Jackson *et al.*, 1996)
MC133L		546		MCV subtype 1	1.5e-11	26/58	44	(Senkevich *et al.*, 1996)
		148		Cotia virus HM2 protein	2.6e-10	21/42	50	(Gershon *et al.*, 1989)
MC131L		513		MCV subtype 1	1.5e-05	18/58	31	(Senkevich *et al.*, 1996)
139L	131739	146	16.3	**16.3k protein**				
A28L	131299	146		VAC	1.7e-103	146/146	100	(Amegadzie *et al.*, 1991a)
A31.5L		146		VAR-BSH (I: A31L)	2.9e-100	141/146	96	(Goebel *et al.*, 1990)
		140		Myxoma virus	1.3e-55	30/52	57	(Shchelkunov *et al.*, 1995)
		140		Cotia virus HM3 protein	3.3e-55	30/49	61	(Jackson *et al.*, 1996)
MC134L		141		MCV subtype 1	1.0e-53	31/52	59	(Gershon *et al.*, 1989)
		143		Amsacta moorei poxvirus	2.0e-14	16/36	44	(Senkevich *et al.*, 1996)
140L	132657	305	35.4	**RNA pol subunit rpo35**				(Hall and Moyer, 1991)
A29L	131740	305		VAC	3.6e-215	304/305	99	(Amegadzie *et al.*, 1991a)
A32L		305		VAR-BSH	7.5e-211	297/305	97	(Goebel *et al.*, 1990)
MC135L		303		MCV subtype 1	7.0e-98	51/103	49	(Shchelkunov *et al.*, 1995)
		126		Cotia virus	2.2e-54	46/61	75	(Senkevich *et al.*, 1996)
141L	132853	77	8.7	**8.7k protein**				(Gershon *et al.*, 1989)
A30L	132620	77		VAC	5.5e-48	77/77	100	(Amegadzie *et al.*, 1991a)
A33L		77		VAR	5.5e-48	77/77	100	(Goebel *et al.*, 1990)
MC136L		67		MCV subtype 1	9.2e-16	18/40	45	(Shchelkunov *et al.*, 1995)
142R	133013	125	14.4	**14.4k protein**				(Senkevich *et al.*, 1996)
A31R	133390	124		VAC	2.0e-84	118/124	95	(Smith *et al.*, 1991)
A34R		140		VAR	1.6e-79	111/114	97	(Goebel *et al.*, 1990)
MC138R		117		MCV subtype 1	6.2e-24	39/98	39	(Shchelkunov *et al.*, 1995)
143L	134169 / 133360	269	30.8	**30.8k protein** ATP/GTP binding motif A				(Senkevich *et al.*, 1997)
A32L		300		VAC	6.4e-190	268/269	99	(Smith *et al.*, 1991)
A35L		270		VAR	1.6e-186	263/269	97	(Koonin *et al.*, 1993)
MC140L		249		MCV subtype 1	7.6e-95	58/94	61	(Goebel *et al.*, 1990)
144R	134287	185	20.6	**EEV glycoprotein**				(Shchelkunov *et al.*, 1995)
A33R	134844	185		VAC	2.1e-124	182/185	98	(Senkevich *et al.*, 1996)
A36R		184		VAR	1.8e-121	103/112	91	(Roper *et al.*, 1996)
		185		Ectromelia	2.8e-113	165/185	89	(Goebel *et al.*, 1990)
145R	134868 / 135374	168	19.6	**EEV glycoprotein virulence factor actin microvilli inducer**				(Shchelkunov *et al.*, 1995)
A34R		168		VAC	1.2e-117	165/168	98	(Roper *et al.*, 1996)
A37R		168		VAR-I	1.7e-117	164/168	97	(Duncan and Smith, 1992a)
		167		FPV ORFs BamHI 2.8.11 hepatic lectins homologs	<0.056	16/66	24	(McIntosh and Smith, 1996)
		199		HS early T-cell activation antigen CD69	0.0038	12/38	31	(Wolffe *et al.*, 1997)
MC143R		159		MCV subtype 1 17 matches to lectins	0.080	12/48	25	(Goebel *et al.*, 1990)
146R	135418	176	20.0	**20.0k protein**				(Tomley *et al.*, 1988)
A35R	135948	176		VAC	1.4e-126	176/176	100	(Hamann *et al.*, 1993)
A38R		60		VAR-I	2.9e-37	57/60	95	(Senkevich *et al.*, 1997)
MC145R		233		MCV subtype 1	1.2e-07	18/55	32	(Smith *et al.*, 1991)
147R	136015 / 136641	208	23.8k	**EEV membrane protein virulence factor**				(Goebel *et al.*, 1990)
A36R		221		VAC	2.8e-143	140/141	99	(Shchelkunov *et al.*, 1995)
A39R		216		VAR 19 matches to asn/ser-rich proteins	2.1e-89	138/177	77	(Parkinson and Smith, 1994)
148R	136705	263	29.8	**29.8k protein**	<0.41			(Smith *et al.*, 1991)
A37R	137496	263		VAC	6.8e-183	261/262	99	(Goebel *et al.*, 1990)
A40R		68		VAR	4.9e-37	61/67	91	(Shchelkunov *et al.*, 1995)
149L	138589	277	31.5	**31.5k protein**				(Goebel *et al.*, 1990)
A38L	137756	277		VAC	9.3e-198	274/277	98	(Shchelkunov *et al.*, 1995)
A41L		277		VAR	1.6e-187	259/277	93	(Amegadzie *et al.*, 1991a)
		303		Rattus norvegicus CD47	3.9e-24	23/86	26	(Goebel *et al.*, 1990)
		324		MM integrin assoc. protein	1.0e-21	23/86	26	(Shchelkunov *et al.*, 1995)
		323		human CD47 precursor	5.0e-19	28/86	32	(Nishiyama *et al.*, 1997)
150R	138606 / 138857	83	9.4	**semaphorin-like protein (f1)**				(Lindberg *et al.*, 1993)
A39R		403		VAC	8.0e-46	73/76	96	(Campbell *et al.*, 1992)
A42R		74		VAR-I	8.6e-44	67/71	94	(Kolodkin *et al.*, 1993)
151R	139163 / 139795	210	23.9	**semaphorin-like protein (f2)**				(Goebel *et al.*, 1990)
A39R		403		VAC	3.0-147	209/210	99	(Shchelkunov *et al.*, 1995)
A43R		139		VAR (I:A44R)	1.8e-68	91/105	86	(Kolodkin *et al.*, 1993)
		653		semaphorin-like protein Alcelaphine herpesvirus 37 matches to semaphorin	1.7e-20	29/79	36	(Goebel *et al.*, 1990) (Shchelkunov *et al.*, 1995) (Ensser and Fleckenstein, 1995)

TABLE 1—*Continued*

ORF[a] left	START / STOP terminal	AA[b] region:	kDa[c]	name / (putative) function / homologies[d]	BLAST[e] expect	BLAST[e] AA id	HSS[f] (%)	references
				/collapsin gene family				
152R	139821 140327	168	19.4	NK cell receptor homolog lectin-like protein				(Scheiflinger et al., unpubl.)
A40R		168		VAC				(Smith *et al.*, 1991)
A45R		61		VAR-I (BSH: A43.5R)	6.6e-97	134/137	97	(Goebel *et al.*, 1990)
		233		HS natural killer (NK) cell protein group 2-A, B	9.6e-36	54/59	91	(Shchelkunov *et al.*, 1995)
		240		HS type II membrane protein	4.5e-11	20/74	27	(Houchins *et al.*, 1991)
		182		MM NK cell receptor	6.9e-11	16/36	44	(Adamkiewicz *et al.*, 1994)
		179		HS CD 94	5.5e-09	16/36	44	(Giorda *et al.*, 1992)
				127 matches to lectins including NK cell surface proteins and snake venoms	1.7e-07	11/29	37	(Chang *et al.*, 1995a)
153L	141025	219	25.1	25.1k protein				
A41L	140366	219		VAC				(Smith *et al.*, 1991)
A44L		218		VAR-BSH (I:A46L)	1.9e-158	218/219	99	(Goebel *et al.*, 1990)
		244		VAC B29R/C23L	1.4e-152	152/159	95	(Shchelkunov *et al.*, 1995)
		258		Rabbit fibroma virus T1	0.0076	12/53	22	(Goebel *et al.*, 1990)
					0.057	13/49	26	(Upton *et al.*, 1987)
154R	141197 141583	128	14.5	profilin-like protein				(Blasco *et al.*, 1991)
A42R		133		VAC				(Smith *et al.*, 1991)
A47R		133		VAR-I (BSH:A45R)	1.2e-87	85/87	97	(Goebel *et al.*, 1990)
		140		HS profilin	1.4e-85	82/87	94	(Shchelkunov *et al.*, 1995)
				10 matches profilin family	2.2e-23	19/45	42	(Kwiatkowski and Bruns, 1988)
155R	141621 142193	190	22.1	class I membrane glycoprotein				(Smith *et al.*, 1991)
A43R		194		VAC				(Duncan and Smith, 1992b)
A48R		195		VAR-I (BSH:A46R)	1.5e-137	162/164	98	(Goebel *et al.*, 1990)
		51		HS leukocyte antigen	1.9e-128	101/109	92	(Shchelkunov *et al.*, 1995)
					0.096	7/23	30	X79517
156R	142201 142437	78	8.8	8.8k protein				(Smith *et al.*, 1991)
		78		VAC-WR SalF6R	3.9e-45	78/78	100	(Smith *et al.*, 1991)
		258		rabbit myosin heavy chain	0.00048	13/39	33	A02985
				144 matches to various asp/glu/lys-rich proteins				
157L	143577 142537	346	39.4	3ß-hydroxysterold dehydrogenase (3ß-HSD)				(Moore and Smith, 1992)
A44L		346		VAC				(Blasco *et al.*, 1991)
A47L		210		VAR-BSH (I: A49L)	4.5e-249	342/346	98	(Goebel *et al.*, 1990)
MC152R		354		MCV subtype 1	1.1e-136	185/195	94	(Shchelkunov *et al.*, 1995)
		369		FPV	8.2e-104	123/272	45	(Senkevich *et al.*, 1996)
				matches to dihydroflavonol reductases, cholesterol dehydrogenases, UDP-galactose-4-epimerases	3.1e-83	33/85	38	(Skinner *et al.*, 1994)
					>2.8e-05			(Baker and Blasco, 1992)
158R	143624 143989	121	13.3	superoxide dismutase-like protein				(Blasco *et al.*, 1991)
A45R		125		VAC				(Smith *et al.*, 1991)
A51R		125		VAR-I BSH A48R	2.1e-82	94/96	97	(Goebel *et al.*, 1990)
				117 matches with superoxide dismutase family	1.1e-82	93/96	96	(Shchelkunov *et al.*, 1995)
					<0.027			
159R	143979	241	27.6	27.6k protein				(Smith *et al.*, 1991)
A46R	144701	214		VAC	9.6e-167	238/240	99	(Goebel *et al.*, 1990)
A52R		240		VAR-I (BSH: A49R)	5.6e-164	233/240	97	(Shchelkunov *et al.*, 1995)
160L	145465	238	27.6	27.6k protein				(Goebel *et al.*, 1990)
J1L	144749	244		VAR	5.1e-146	114/127	89	(Shchelkunov *et al.*, 1995)
A47L		244		VAC	8.2e-135	121/127	95	(Goebel *et al.*, 1990)
				integrin lipid binding motif				(Smith *et al.*, 1991)
161R	145564	204	23.2	thymidylate kinase				(Smith *et al.*, 1991)
A48R	146178	204		VAC	5.2e-140	204/204	100	(Goebel *et al.*, 1990)
J2R		205		VAR	1.1e-137	161/165	97	(Shchelkunov *et al.*, 1995)
				16 matches to thymidylate kinase family	<0.49			
162R	146202	162	18.8	18.8k protein				(Smith *et al.*, 1991)
A49R	146690	162		VAC	6.0e-106	159/162	98	(Goebel *et al.*, 1990)
J3R		162		VAR	2.4e-103	154/162	95	(Shchelkunov *et al.*, 1995)
163R	146722	552	63.5	DNA ligase				(Kerr and Smith, 1989)
A50R	148380	552		VAC	0.0	547/552	99	(Goebel *et al.*, 1990)
J4R		552		VAR-I	0.0	537/552	97	(Shchelkunov *et al.*, 1995)
		922		HS DNA ligase III	2.1e-235	102/165	61	(Wei *et al.*)
		559		shope fibroma ligase	9.9e-213	95/200	47	(Parks *et al.*, 1994)
		564		FPV ligase	3.0e-195	101/170	59	(Skinner *et al.*, 1994)
				31 matches mainly to DNA ligase family	<0.029			
164R	148426	310	34.9	34.9k protein				(Antoine *et al.*, 1996)
A51R	149358	334		VAC	1.5e-217	267/274	97	(Goebel *et al.*, 1990)
J5R		334		VAR	9.1e-208	251/274	91	(Shchelkunov *et al.*, 1995)

TABLE 1—*Continued*

ORF[a] left	START terminal / STOP terminal	AA[b] region:	kDa	name / (putative) function / homologies	BLAST[d] expect	BLAST AA id	HSS[e] (%)	references
				fusion of *A51R*/*A55R* ORFs				(Antoine *et al.*, 1996)
165R	149416	315	34.8	**hemagglutinin**				
A56R	150363	315		VAC				(Shida, 1986)
J9R		313		VAR-I (BSH:J7R)	1.8e-211	312/315	99	(Goebel *et al.*, 1990)
		310		raccoonpox	4.3e-178	183/238	76	(Shchelkunov *et al.*, 1995)
				124 matches to various proteins	1.5e-91	74/104	71	(Cavallaro and Esposito, 1992)
					<0.34			
166R	150659	97	11.4	**guanylate kinase (f)**				
A57R	150952	151		VAC				(Smith *et al.*, 1991)
J10R		151		VAR (BSH:J8R)	3.2e-62	94/97	96	(Goebel *et al.*, 1990)
		198		MM guanylate kinase	2.2e-57	88/97	90	(Shchelkunov *et al.*, 1995)
		197		HS guanylate kinase	4.3e-24	39/91	42	(Brady *et al.*, 1996)
				21 matches mainly to guanylate kinases	2.8e-20	35/91	38	(Brady *et al.*, 1996)
					<0.20			
167R	151103	300	34.3	**serine/threonine protein kinase**				(Howard and Smith, 1989)
	152005							(Banham and Smith, 1992)
B1R		300		VAC				(Lin *et al.*, 1992)
B1R		300		VAR-I	7.1e-215	298/300	99	(Goebel *et al.*, 1990)
		283		VAC B12R	2.7e-210	289/300	96	(Shchelkunov *et al.*, 1995)
				100 matches mainly to protein kinase family	4.9e-49	27/53	50	(Goebel *et al.*, 1990)
					<0.00031			
168R	152144	96	11.5	**24.6k protein (f1)**				
B2R	152434	219		VAC				
		149		histone H2A pea	8.5e-38	54/60	90	(Goebel *et al.*, 1990)
169R	152289	143	16.1	**24.6k protein (f2)**	0.59	16/50	32	P40281
B2R	152720	219		VAC				(Goebel *et al.*, 1990)
					5.7e-86	124/128	96	(Goebel *et al.*, 1990)
170R	152917	179	20.9	**20.9k protein (f)**				
B3R	153456	124		VAC				
		167		VAC WR	8.2e-33	51/56	91	(Goebel *et al.*, 1990)
		92		VAR-GAR H5R	5.3e-45	51/56	91	(Smith *et al.*, 1991)
					3.4e-06	19/28	67	U18339
171R	153683	177	21.4	**65k ank-like protein virulence factor (f1)**				(Howard *et al.*, 1991)
	154216							(Mossman *et al.*, 1996)
B4R		558		VAC	8.5e-107	151/154	98	(Goebel *et al.*, 1990)
B6R		558		VAR-I (BSH:B5R)	1.7e-98	140/154	90	(Shchelkunov *et al.*, 1995)
172R	154107	409	47.7	**65k ank-like protein virulence factor (f2)**				(Howard *et al.*, 1991)
	155336							(Mossman *et al.*, 1996)
B4R		558		VAC	2.4e-283	195/201	97	(Goebel *et al.*, 1990)
B6R		558		VAR-I (BSH:B5R)	2.3e-270	185/201	92	(Shchelkunov *et al.*, 1995)
		483		MYX M-T5 protein	5.5e-10	19/57	33	(Mossman *et al.*, 1996)
		1765		MM ankyrin 3	9.7e-10	22/54	40	(Peters *et al.*, 1995)
		516		orf virus	1.8e-09	16/47	34	U34774
		574		VAC B18R	3.3e-09	11/23	47	(Goebel *et al.*, 1990)
		574		VAR-I B19R	3.6e-09	19/72	26	(Shchelkunov *et al.*, 1995)
		882		HS KIAA0379	5.1e-09	20/52	38	AB002377
		668		CPX host range gene	1.7e-08,	14/47	29	(Spehner *et al.*, 1988)
		237		VAC WR hr gene	2.8e-08	15/47	31	(Kotwal and Moss, 1988a)
		472		VAC M1L	5.1e-07	23/81	28	(Goebel *et al.*, 1990)
		474		CPX O1L	8.7e-07	22/61	36	(Safronov *et al.*, 1996)
		446		VAR O1L	8.8e-07	23/81	28	(Shchelkunov *et al.*, 1995)
		437		CPX D1L	1.7e-06	8/27	29	(Safronov *et al.*, 1996)
		634		VAC C9L	7.8e-05			(Goebel *et al.*, 1990)
				159 matches including ankyrin proteins				
173R	155424	317	35.1	**ps/hr protein/ EEV gp42**				(Takahashi-Nishimaki *et al.*, 1991)
	156377							(Engelstad *et al.*, 1992)
B5R		317		complement control protein				(Isaacs *et al.*, 1992)
B7R		317		VAC	1.6e-232	312/317	98	(Goebel *et al.*, 1990)
		259		VAR-I (BSH:B6R)	7.1e-220	294/316	93	(Shchelkunov *et al.*, 1995)
				CPX D17L	2.1e-12	16/52	30	(Safronov *et al.*, 1996)
				186 matches to complement control protein family	<7.7e-05			
174R	156474	173	20.2	**20.2k protein**				
B6R	156995	173		VAC				
B7R		65		VAR-BSH (I:B8R)	1.5e-121	173/173	100	(Goebel *et al.*, 1990)
		685		NAD-protein ADP ribosyl-transferase phage T4	6.0e-40	62/65	95	(Shchelkunov *et al.*, 1995)
					0.56	17/56	30	SXBPT4
175R	157033	177	20.7	**20.7k protein**				
B7R	157566	182		VAC				
		184		VAC C8L	7.8e-129	95/108	87	(Goebel *et al.*, 1990)
		182		CPX D12L	0.16	9/44	20	(Goebel *et al.*, 1990)
				EF-hand calcium-binding domain	0.49	8/36	22	(Safronov *et al.*, 1996)
176R	157621	226	26.0	**31k interferon-gamma receptor (f)**				(Upton *et al.*, 1992)
	158301							(Alcami and Smith, 1995)
B8R		272		VAC	3.3e-164	116/123	94	(Goebel *et al.*, 1990)
B8R		266		VAR-BSH (I:B9R)	3.0e-153	111/123	90	(Shchelkunov *et al.*, 1995)
		266		ECT	2.6e-151	110/123	89	(Mossman *et al.*, 1995b)
		274		swinepox C6	3.2e-09	12/31	38	(Massung *et al.*, 1993)

TABLE 1—*Continued*

ORF[a]	START STOP	AA[b]	kDa[c]	name / (putative) function / homologies[e]	BLAST[d] expect	BLAST[e] AA id	HSS[f] (%)	references
left	terminal	region:						
177R	158458	72	8.3	8.3k protein				
B9R	158676	77		VAC	3.0e-49	60/60	100	(Goebel *et al.*, 1990)
		240		capripox T4 protein	1.2e-09	16/44	36	M28823
		237		shope fibroma virus	0.0057	15/50	30	F43692
178R	158639	158	17.9	17.9k protein				
B10R	159115	166		VAC	4.7e-110	146/146	100	(Goebel *et al.*, 1990)
		530		swinepox VC04	0.040	13/42	30	(Massung *et al.*, 1993)
		689		kelch protein D. melanogaster	0.14	12/54	27	(Xue and Cooley, 1993)
								(Senkevich *et al.*, 1993b)
179R	159187	74	8.5	8.5k protein				
B11R	159411	88		VAC	9.2e-43	70/73	95	(Goebel *et al.*, 1990)
				177 matches to glu/asn rich proteins				
180R	159478	283	33.3	protein kinase				(Howard and Smith, 1989)
B12R	160329	283		VAC	1.8e-207	282/283	99	(Goebel *et al.*, 1990)
B12R		134		VAR-1	8.7e-26	31/54	57	(Shchelkunov *et al.*, 1995)
		300		VAC B1R	1.7e-54	26/53	49	(Goebel *et al.*, 1990)
		300		VAR-1 B1R	7.7e-53	25/53	47	(Shchelkunov *et al.*, 1995)
				120 matches mainly to protein kinase family	<0.34			
181R	160437 160787	116	13.0	ICE inhibitor / SPI-2 (f1)				(Kotwal and Moss, 1989) (Smith *et al.*, 1989)
B13R				VAC				(Ray *et al.*, 1992)
B13R		116		VAR-1 (BSH:B12R)	3.0e-72	111/116	95	(Goebel *et al.*, 1990)
		344		CPX crmA	2.7e-69	105/114	92	(Shchelkunov *et al.*, 1995)
		341		VAC C12L (SPI-1)	2.8e-39	66/100	66	(Pickup *et al.*, 1986)
		353		Ectromelia serpin	2.1e-23	25/34	73	(Goebel *et al.*, 1990)
		344		rabbitpox SPI-1	9.2e-23	24/34	70	(Senkevich *et al.*, 1993b)
		357		CPX SPI-1	5.5e-22	25/34	73	(Ali *et al.*, 1994)
		355		VAR-1 B25R (BSH:B21R)	1.4e-21	25/36	69	(Ali *et al.*, 1994)
		372		CPX serpin-like protein	1.7e-21	25/34	73	(Shchelkunov *et al.*, 1995)
		372		135 matches mainly to serpins	1.7e-36 <0.12	25/36	69	(Ali *et al.*, 1994)
182R	160762	222	24.9	ICE inhibitor/SPI-2 (f2)				see above
B14R	161430	222		VAC	6.2e-158	218/222	98	(Goebel *et al.*, 1990)
		345		VAC WR	9.4e-156	215/221	97	(Kotwal and Moss, 1989)
		345		rabbit pox SPI-2	1.6e-153	211/221	95	(Ali *et al.*, 1994)
		341		CPX crmA	4.5e-148	203/220	92	(Pickup *et al.*, 1986)
B13R		344		VAR-1 (BSH:B12R)	1.5e-146	203/220	92	(Shchelkunov *et al.*, 1995)
				309 matches see above	<1.3e-21			
183R	161506	143	16.7	16.7k protein				(Smith and Chan, 1991)
B15R	161937	149		VAC	3.6e-105	97/98	98	(Goebel *et al.*, 1990)
B14R		149		VAR-1 (BSH:B13R)	9.1e-104	95/98	96	(Shchelkunov *et al.*, 1995)
		153		VAR-1 D1L (BSH:D2L)	8.8e-31	25/52	48	(Shchelkunov *et al.*, 1995)
		181		VAC C16L/B22R	1.0e-26	25/52	48	(Goebel *et al.*, 1990)
		159		capripox T3A	1.4e-17	17/42	40	(Gershon and Black, 1989a)
		151		rabbit fibroma T3A	2.6e-07	17/44	38	(Upton *et al.*, 1987)
		190		VAC A52R	0.073	10/28	35	(Goebel *et al.*, 1990)
		149		VAC WR K7R	0.21	7/22	31	(Boursnell *et al.*, 1988)
		149		VAR-1 C4R	0.30	7/22	31	(Shchelkunov *et al.*, 1995)
		161		CPX M6R	0.51	7/22	31	(Safronov *et al.*, 1996)
X	184R	162021 163001	326	36.6	Interleukin-1ß receptor (IL-1ßR)				(Alcami and Smith, 1992) (Spriggs *et al.*, 1992)
		326		VAC-WR B15R	2.8e-229	323/326	99	(Smith *et al.*, 1991)
		326		CPX B16	2.3e-217	306/326	93	(Spriggs *et al.*, 1992)
	B16R	290		VAC	4.4e-202	287/290	98	(Goebel *et al.*, 1990)
	B17R	69		VAR-1 (BSH:deleted)	8.1e-38	59/68	86	(Shchelkunov *et al.*, 1995)
		296		HS type II IL-1 receptor	1.7e-36	28/75	37	U64094
				271 matches mainly to IL-1 receptors, growth factor receptors and Ig family proteins	<0.011			
X	185L	164069	340	39.6	39.6k protein				
	B17L	163047	340		VAC	4.8e-248	335/340	98	(Goebel *et al.*, 1990)
	B15L		340		VAR-BSH (I:B18L)	2.7e-241	325/340	95	(Shchelkunov *et al.*, 1995)
X	186R	164209	574	68.0	68k ank-like protein				(Smith *et al.*, 1991)
	B18R	165933	574		VAC	0.0	560/574	97	(Goebel *et al.*, 1990)
	B19R		574		VAR-1 (BSH:B16R)	0.0	539/574	93	(Shchelkunov *et al.*, 1995)
					100 matches mainly to poxvirus ankyrin proteins	<0.53			
	187R	165999 166703	234	27.5	surface antigen, IFN-alpha/beta receptor (f)				(Ueda *et al.*, 1990) (Symons *et al.*, 1995)
	B19R		353		VAC (WR:B18R)	1.4e-163	218/233	93	(Colamonici *et al.*, 1995)
	B20R		354		VAR-1 (BSH:B17R)	1.53-149	111/133	83	(Goebel *et al.*, 1990)
			569		HS interleukin-1 receptor	0.0051	15/43	34	(Shchelkunov *et al.*, 1995)
					28 matches mainly to IL-1 receptors	<0.53			(McMahan *et al.*, 1991)
	188R	167202	70	8.2	8.2k protein (f)				

TABLE 1—*Continued*

ORF[a]	START STOP	AA[b]	kDa[c]	name / (putative) function / homologies[g]	BLAST[d] expect	BLAST[e] AA id	HSS[f] (%)	references
left	terminal	region:						
B22R	167414	1897		VAR-BSH (I:B26R)	9.9e-23	31/38	81	(Shchelkunov *et al.*, 1995)
189R	167897	188	21.7	21.7k protein				
B22R	168463	181		VAC B22R/C16L	2.9e-111	95/104	91	(Goebel *et al.*, 1990)
D1L		153		VAR-I(BSH:D2L)	1.2e-88	66/71	92	(Shchelkunov *et al.*, 1995)
		149		VAC B15R	7.2e-19	25/52	48	(Goebel *et al.*, 1990)
		159		capripox T3A	8.0e-05	15/45	33	(Gershon and Black, 1989a)
		151		VAC C6L	0.25	12/46	26	(Goebel *et al.*, 1990)
		156		VAR (I:D9L;BSH:D12L)	0.26	12/46	26	(Shchelkunov *et al.*, 1995)
190R/	168531	233	26.9	45k ank-like protein (f2)				
004L	169232							
B23R		386		VAC (C17L/B23R)	6.2e-159	110/110	100	(Goebel *et al.*, 1990)
D1L		91		VAR-BSH	9.1e-31	46/49	93	(Shchelkunov *et al.*, 1995)
		669		CPX host range	1.1e-13	22/50	44	(Spehner *et al.*, 1988)
		452		VAR-I D6L (BSH:D8L)	1.7e-11	21/50	42	(Shchelkunov *et al.*, 1995)
		574		VAC B18R (WR: B17R)	1.2e-05	22/73	30	(Shchelkunov *et al.*, 1995)
		574		VAC C9L	8.6e-05	22/73	30	(Goebel *et al.*, 1990)
		634		VAR-I G1R	0.00011	11/24	45	(Kotwal and Moss, 1988a)
		585		orf virus	0.00013	22/74	29	(Shchelkunov *et al.*, 1995)
		516		VAR-I D7L (BSH:D10L)	0.0088	15/49	30	(Sullivan *et al.*, 1995b)
		153			0.014	12/28	42	(Shchelkunov *et al.*, 1995)
191R/	169309	102	12.1	45k ank-like protein (f1)				
003L	169617							
B23R		386		VAC C17L/B23R	1.3e-39	62/63	98	(Goebel *et al.*, 1990)
192R/	170305	176	19.7	secr. TNF receptor (f)				
002L	170835	355		CPX crmB				(Upton *et al.*, 1991a)
G2R		348		VAR-BSH	5.1e-71	76/83	91	(Hu *et al.*, 1994)
		326		Myxoma virus T2	1.0e-66	73/83	87	(Shchelkunov *et al.*, 1995)
		325		Rabbit fibroma Virus T2	4.9e-30	21/37	56	(Upton *et al.*, 1991a)
		202		CPX C4L	1.8e-28	17/36	47	(Upton *et al.*, 1987)
B25R		346		HS TNF receptor	8.7e-15	30/51	58	(Heller *et al.*, 1990)
		259		VAC (*C19L/B25R*)	1.9e-08	14/26	53	(Safronov *et al.*, 1996)
		277		human CD40L receptor	0.00026	16/19	84	(Goebel *et al.*, 1990)
				30 matches to TNF receptors and surface proteins	0.0015	11/24	45	(Stamencovic *et al.*, 1989)
					<0.39			
193R/	171267	136	14.9	35k major secr. protein chemokine receptor (f)				(Patel *et al.*, 1990)
001L	171677							(Graham *et al.*, 1997)
B29R		244		VAC (*C23L/B29R*)	6.0e-57	41/42	97	(Goebel *et al.*, 1990)
G5R		253		VAR-I	8.9e-51	46/49	93	(Shchelkunov *et al.*, 1995)
		246		CPX ORF B	5.6e-49	40/42	95	(Hu *et al.*, 1994)
		258		SFV T1 protein	2.5e-20	23/42	54	(Upton *et al.*, 1987)
		260		Myxoma virus T1/35kDa	1.5e-14	21/42	50	(Graham *et al.*, 1997)

[a] Open reading frame coding for at least 65 amino acids (for exceptions see text); minor ORFs located in reverse orientation within large ORFs or ORFs located in the repeat regions of the ITRs (see text) are not listed; the MVA ORFs (boldface), listed consecutively as appearing in the genome, and homologs in the Copenhagen strain (in italics), in the variola strains and in the molluscum contagiosum, are listed in this row. Split ORFs are boxed.

[b] Number of deduced amino acids (AA) encoded within an ORF.

[c] Predicted M_r (kDa) for the unmodified protein.

[d] The lowest Poisson probability determined by the BLAST search (Altschul *et al.*, 1990). The Expect value of 0.0 indicates a probability of zero that an alignment occurs by chance; low Expect values correspond to high homology and vice versa.

[e] Amino acid identity (AA id) of first high-scoring segment pair in the BLASTp protocol.

[f] Amino acid identity of first high-scoring segment pair (HSS)%.

[g] Homologies based on searching PIR and SWISS-PROT databases (BLASTp nr).

[h] Duplicated ORFs located in ITRs.

Fragment; complete homologous ORF present in related poxvirus (see reference).

[i] Variola India (I) or variola Bangladesh (BSH) sequences; in cases where the variola sequences are not identical, the variola strain first appearing in the blast search protocol is listed.

[k] ank, ankyrin.

[l] HS, homo sapiens.

[m] MM, *Mus musculus*.

structures reflecting the natures of their DNA templates (not shown). For this family of hypothetical proteins, no significant homologies were found except for the homology to the ORF G4R, the last ORF present in the repeat region of the right terminus of the Brazilian alastrim variola minor virus strain Garcia-1966 (Massung *et al.*, 1996). Minor ORFs located in the reverse orientation within large ORFs and minor overlapping ORFs were analyzed but no homologies except for the CPN homologs (Goebel *et al.*, 1990) were found; these ORFs are also not listed in Table 1. Although all MVA ORFs were named systematically according to their appearance in the genome, the established ORF nomenclature of the CPN strain (Goebel *et al.*, 1990) was used in this report for the homologous MVA ORFs unless otherwise indicated.

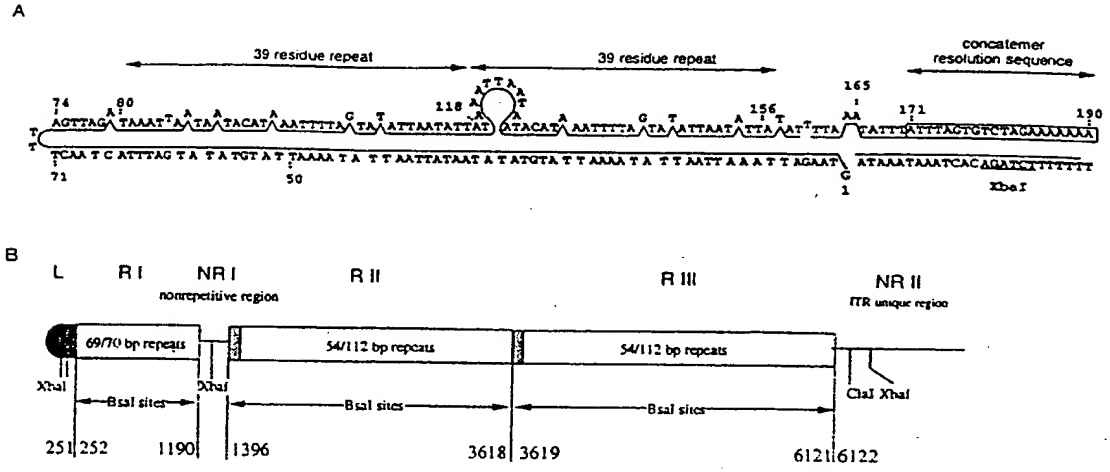

FIG. 1. (A) Structure of the terminal hairpin loops of MVA. The program DNAsis (Hitachi, inc.) was used to calculate the most stable basepairing. The location of the 39 residue tandem repeats and the concatemer resolution sequence (Merchlinsky and Moss, 1989) are indicated by arrows. The G-residue of the first mismatched basepair was defined as residue 1. Thus, the database entry starts with this G-residue and proceeds leftward around the hairpin. (B) Repeat structure of the termini. The 9.8-kb-sized inverted terminal repeat (ITR) consists of six sequence elements, the terminal hairpin loop (L), the repeat regions I–III (RI–RIII), and the nonrepeated regions I and II (NRI–II). Restriction sites critical for cloning and characterization of the repeats (*ClaI*, *XbaI*, *BsaI*) are indicated; the recognition sequence of the enzyme *BsaI* is part of the basic repeat unit and thus occurs frequently within the repeat regions. The numbers indicate the positions of the sequence elements within the MVA genomic sequence.

Structure of termini

The linear double-stranded DNA genome of poxviruses has covalently closed termini; the telomeric regions are identical but inverted in sequence (review: Moss, 1996). Within the poxviruses, ITRs are variable in sequence and length; the CPN and the shope fibrome ITRs, for instance, are 12 kb in size (Cabirac *et al.*, 1985; Goebel *et al.*, 1990) and encode for up to 12 ORFs, while the variola Bangladesh (VAR-BSH) ITR is 725 bp long and does not include ORFs larger than 65 amino acids (Massung *et al.*, 1994). In MVA the ends of the genome contain 9.8-kb ITR that are identical up to positions 9809 and 168,280.

To characterize the incompletely base-paired terminal hairpin loops, which are characteristic for poxviral ITRs (Moss, 1996), telomeric RNA transcribed from the concatemer resolution sequence (Hu and Pickup, 1991) was converted into cDNA and sequenced. The MVA terminal hairpin loops are 165 residues in size, differing from the usual size of about 100 bases. The loop contains a perfect 39-nucleotide-long tandem repeat at positions 80–118 and 119–156, not present in the CPN strain (Fig. 1A). Similar to the CPN sequence, the first base of the MVA genome was defined to be the first mismatched residue of the hairpin loop (see below). Thus, the database entry, consisting of the upper DNA strand in 5′-3′ orientation, starts with this G-residue and proceeds leftward around the 165 bases of the hairpin (see Fig. 1) and proceeds to the last base 177,923, the junction to the right hairpin loop, that is formally provided by the lower DNA strand and therefore not repeated in the database entry. The left concatemer resolution sequence is located adjacent to the hairpin (positions 171–190; see Fig. 1); the right concatemer resolution sequence is located at positions 177,899–177,918.

The structure of the repeated elements at both ends of the genome are depicted in Fig. 1B. Six segments were identified within the ITR; three regions of tandem repeats (RI, RII, and RIII) are located next to the hairpin loop (L) with an intervening nonrepetitive segment NRI. The inner part of the ITR consists of another nonrepetitive sequence NRII. Adjacent to the hairpin loop, an outer bloc of 13 tandem repeats (RI; Fig. 1B) is located from bases 252 to 1190; the equivalent region in the CPN strain is similarly organized but about three times larger. The RI region is composed of two types of different repeated elements, the 69- and the 70-bp repeat. Southern blot analyses confirmed that this region is responsible for a microheterogenicity of the terminal 1.1-kb *XbaI* fragment (not shown). The outer bloc is followed by the nonrepetitive region NRI (positions 1191 to 1395). Interestingly, a sequence stretch starting at position 1396 is repeated in position 3619 and nowhere else in the genome. Regions RII (positions 1396–3618) and RIII (positions 3619–6121) are very similar to each other and obviously the result of a duplication. Thus, MVA has two inner blocs, RII and RIII, 2.2 and 2.5 kb in length, respectively. The tandem repeats of the RII–RIII region start at base 1500 and end at 6121. RII and RIII contain clusters from one to seven copies of a conserved 54-bp repeat element also found in the CPN strain. Between these clusters, repeat elements similar to the 54-bp repeat element, but with variations in the sequence and length, are found. All repeats in RI, RII, and RIII have a 14-bp sequence (AAGAGAGAAAGAGA) in common, which is repeated 77

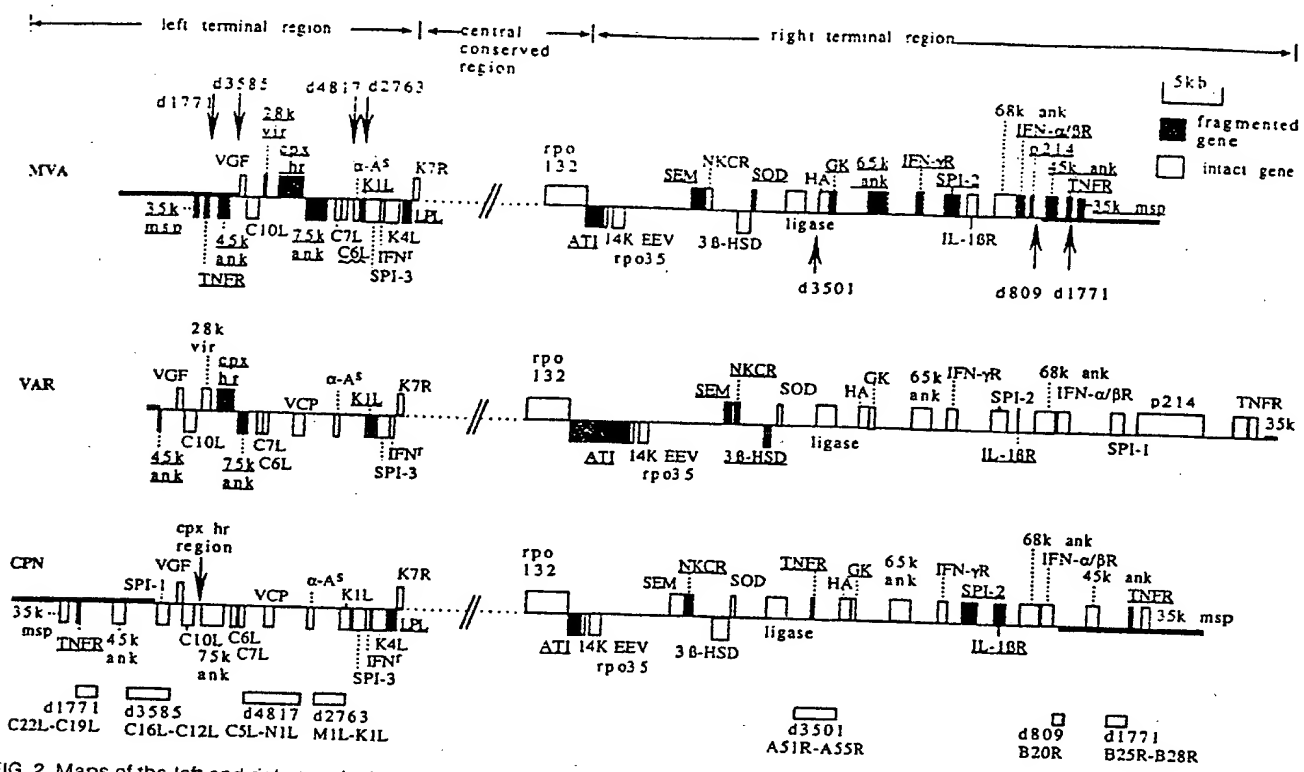

FIG. 2. Maps of the left and right terminal regions of the vaccinia strains MVA and Copenhagen (CPN) and variola virus (VAR). A selection of genes involved in host–virus interaction is shown. Filled boxes indicate fragmented and open boxes structurally intact genes; names of fragmented genes are underlined. Deletion >150 bp in MVA relative to the CPN strain are indicated by bars located below the CPN map; the numbers refer to the size of the deletion (d) in basepairs. Vertical arrows in the MVA map indicate the positions of the deletions. ORFs in the left terminal region: 35k msp, 35-kDa major secreted protein, chemokine receptor (C23L); TNFR, tumor necrosis factor receptor (C22L); 45k ank, 45-kDa ankyrin-like protein (C17L); VGF, vaccinia growth factor (C11R); 28k vir, 28-kDa virulence factor (VAR-BSH D6R); cpx hr, 77-kDa cowpox host range protein (VAR ORFs D5L and D6L); SPI-1, serpin SPI-1 (C12L); 75k ank, 75-kDa ankyrin-like protein (C9L); C6L, 18.2k protein of unknown function; C7L, host range protein; VCP, 35-kDa vaccinia complement control protein (C3L); α-A^s, alpha-amanitin-sensitive protein (N2L); K1L, host range gene; SPI-3, serpin SPI-3 (K2L); IFN^r, interferon resistance (K3L); LPL, lysophospholipase-like gene (K5L). ORFs in the right terminal region: rpol32 (35), 132 (35)-kDa subunit of the RNA polymerase (A24R and A29L); ATI, cowpox A-type inclusion body region (A25L); 14k EEV, 14-kDa extracellular enveloped virus protein (A27L); SEM, semaphorin-like protein (A39R); NKCR, natural killer cell receptor homolog (A40R); 3β-HSD, 3β-hydroxysteroid dehydrogenase (A44L); SOD, superoxide dismutase-like protein (A45R); HA, hemagglutinin (A56R); GK, guanylate kinase (A57R); 65k ank, 65-kDa ankyrin-like protein (B4R); IFN-γR, interferon-γ receptor (B8R); SPI-2, serpin SPI-2 (B13R); IL-1βR, interleukin-1β receptor(B16R); 68k ank, 68-kDa ankyrin (B18R); IFN-α/βR, interferon-α/β receptor (B19R); p214, variola 214-kDa protein (VAR B22R). Boldface horizontal lines indicate the extent of the inverted terminal repeats. Dotted lines help to identify the respective gene locus.

times, found for the first time at position 359 and ending the last time at position 5972.

Fragmented and deleted ORFs in the left terminal genomic region

The genomes of orthopoxviruses may be subdivided into a left terminal genomic region, spanning the region from the left hairpin loop to the lysophospholipase (LPL)-like gene (K6L), with the central conserved region including ORFs K7R to A24R (encoding a hypothetical 17.5-kDa protein and the RNA polymerase subunit rpo132, respectively) and the right terminal region extending from the A-type inclusion body (ATI) region (A25L) to the right hairpin loop (Massung *et al.*, 1994). The majority of ORFs in the left terminal genomic region of MVA are deleted, fragmented, or truncated (see Fig. 2 and Table 1). The first gene in the vaccinia CPN strain, C23L encoding the 35-kDa major secreted protein (Patel *et al.*, 1990), recently shown to encode a chemokine binding protein expressed by several orthopoxvirus strains (Graham *et al.*, 1997), is disrupted in MVA. Fragmented ORFs in the left terminal genomic region further include C19L, encoding a tumor necrosis factor receptor (TNFR) homolog (Hu *et al.*, 1994; Upton *et al.*, 1991a), and C17L, coding for the 45-kDa ankyrin-like protein (Goebel *et al.*, 1990). A region common to MVA and the variola Bangladesh (BSH) strain, but absent in CPN, is the highly fragmented cowpox (CPX) virus host range (hr) gene region. This region includes a fragmented ORF (MVA007R) defined as a virulence factor in ectromelia virus, the 28k virulence factor (Senkevich *et al.*, 1993a), which is also structurally intact in variola and CPX (Table 1), and a 13.7-kDa protein (MVA008L) that is homologous to a variola ORF (BSH-D7L) and to a corresponding ectromelia and CPX ORF.

Alignment of the CPX hr gene region with the corresponding sequence in MVA (not shown) confirmed that an MVA homolog to the CPX hr gene is present, but, due to deletions and frameshifts, is split into five ORFs (MVA009L-013L). An interrupted remnant of the CPX hr gene is also present in the vaccinia WR strain (Kotwal and Moss, 1988a) and in the ectromelia strain Moscow (Chen *et al.*, 1992).

Adjacent to the CPX hr gene homolog is the 75-kDa ankyrin-like protein (75k ank, C9L), which is split into three ORFs in MVA (MVA014L-016L). Furthermore, the neighboring C8L ORF has a 21-nucleotide internal deletion and the secreted 14k virulence factor (N1L) is partially deleted. A further potential virulence factor, which is intact in CPX and ectromelia, but split into the ORFs MVA026L and 27L as well as in the corresponding ORFs K5L and K6L in the CPN strain (see Table 1 and Fig. 2), is the vaccinia LPL homolog, which is homologous to human and bacterial lysophospholipases (R. L. Buller & C. Upton, unpublished).

Four large deletions in the MVA left terminal region relative to the CPN sequence, termed d1771, d3585, d4817, and d2763 (the numbers refer to the size of the deletion in basepairs) include totally or partially the ORFs C22L-C19L, C16L-C12L, C5L-N1L, and M1L-K1L (see lower part of Fig. 2). Among them is a potential virulence factor, the vaccinia complement-binding protein (VCP) that modulates complement activation (Kotwal, 1988; Miller *et al.*, 1997), the serpin SPI-1 (C12L), and the host range gene K1L (see also Altenburger *et al.*, 1989), thought to be necessary for growth of vaccinia in human cells.

Structurally intact ORFs in the left terminal genomic region

Only 8 of 27 listed ORFs are structurally intact in the left terminal region of MVA (see Table 1 and Fig. 2) and presumably encode functional proteins. The intact ORFs encode the vaccinia growth factor VGF (C11R), the C10L and C6L proteins of unknown function, the C7L hr protein, the α-amanitin-sensitive protein (N2L), the serpin SPI-3 (K2L), an interferon resistance protein (K3L) and the protein encoded by ORF K4L. The ORF K4L, encoding a major poxvirus envelope antigen present in various poxviruses except for variola, is highly homologous to a human member of the phospholipase D superfamily (Cao *et al.*, 1997). Although structural integrity of an ORF does not mean expression of a functional protein, the eight intact ORFs in the left terminal region of MVA seem to be the minimal requirement of genes necessary for an efficient vaccinia vaccine strain. In summary, the left terminal genomic region of MVA is unique and includes relatively few intact genes, most of which seem to be involved in host–virus interaction. The region has large deletions but also a large insertion relative to the prototype vaccinia CPN sequence. The presence of the large "cowpox hr-region" and its adjacent genes not present in the CPN strain supports the idea that CPX or a CPX-like virus is the ancestor of vaccinia viruses. This view is further supported by the fact that the vaccinia WR strain (Kotwal and Moss, 1988a) and the ectromelia Moscow strain (Chen *et al.*, 1992; Senkevich *et al.*, 1993a) also enharbor a cowpox hr region including the 28k virulence factor and the adjacent 13.7k protein (or remnants thereof) and interrupted versions of the CPX hr gene (see also Safronov *et al.*, 1996). DNA alignments of the CPX hr genes of the CPX Brighton strain, the vaccinia MVA and WR strains, and the ectromelia Moscow strain revealed a closer relationship of the vaccinia and ectromelia sequences compared to the cowpox sequence (data not shown), again arguing for an ancestral CPX or CPX-like virus as an ancestor of vaccinia and ectromelia virus. Although similar, the mutations in the CPX hr genes of the MVA and the WR strain result in different fragmentations on the protein level excluding a closer relationship of the two strains. Since the CPX hr gene is sufficiently divergent between orthopoxviral strains, it seems to be an excellent candidate for establishing phylogenetic relationships.

Fragmented and mutated genes in the central conserved genomic region

As expected, most of the ORFs in the central conserved region are intact, although amino acid changes, compared to the respective homologs in the CPN and VAR strains, are frequent (see also Table 2). Surprisingly, however, three fragmented ORFs (F5L, F11L, and O1L) were found in the MVA central region, indicating that these genes are nonessential and constitute potential stable insertion sites for foreign genes. In fact, WR strain-based mutants inactivating the F5L, F11L, and O1L loci could be generated, confirming their nonessential character (F. Scheiflinger, unpublished). The ORF F5L, encoding the 36.5k major membrane precursor (Roseman and Slabaugh, 1990), is split into two ORFs in MVA (MVA033L and MVA034L). An array of small ORFs of unknown function, F6L-F8L (MVA 035L-037L), located downstream of F5L, is present in variola and vaccinia strains. Further analysis of corresponding orthopoxviral sequences would be useful to clarify the question of whether this region is the remnant of one large gene.

The second fragmented ORF of the central region, F11L, encoding a 39.7-kDa protein of unknown function, is split into the ORFs MVA040L and MVA041L. In the orf virus strain NZ2, the F11L homolog is an early gene located near the left terminus in the orf virus genome (Sullivan *et al.*, 1995a).

The third ORF in this region that is split into two parts (MVA059L and MVA060L) is O1L, encoding a 77.6-kDa protein of unknown function. This protein contains a leucine zipper and a bipartite nuclear target sequence (Goebel *et al.*, 1990). Two of the nonessential ORFs also have ho-

TABLE 2

Divergent Homologous ORFs Located in the Central Conserved Region of MVA, CPN, and Variola Virus

ORF name			Amino acids	Deletion/insertion >2aa/function or homology
MVA[a]	CPN	VAR[b]	MVA/CPN/VAR	
029L	F1L	C5L	222/226/237	12nt del; ukn
031L	F3L	C7L	476/480/161	12nt del; envelope antigen
032L	F4L	C8L	319/319/333	Ribonucleotide reductase
033L	F5L	C9L	97/321/348	Frame shift; truncation
034L	F5L	C9L	218/321/348	Membrane protein precursor
036L	F7L	C11L	80/92/79	36nt del (lys-asn repeats)
040L	F11L	C15L	84/354/354	Multiple deletions; truncation
041L	F11L	C15L	100/354/354	ukn
052R	E5R	E5R	331/331/341	30nt del; ukn
054R	E7R	E7R	166/166/60	17k myristylprotein; ukn
055R	E8R	E8R	273/273/273	Deletion in promoter region; ukn
059L	O1L	Q1L	152/666/666	19nt, 25nt del; fragmentation
060L	O1L	Q1L	405/666/666	Leu-zipper pattern; ukn
085R	J1R	L1R	153/153/159	Dimeric virion protein; ukn
095R	H5R	I5R	203/203/220	VLTF-4
100R	D3R	F2R	233/237/237	12nt del; structural 27k protein
104R	D7R	F7R	161/161/157	RNA pol subunit
115L	A4L	A5L	272/282/271	27nt del; core protein
120L	A9L	A10L	94/99/95	15nt del; ukn
123L	A12L	A13L	187/192/189	15nt del; virion protein
133R	A22R	A23R	187/176/187	ATG mutated in CPN; ukn
135R	A24R	A25R	1155/1164/1164	"Minor" ATG mutated; rpol132

[a] Split ORFs are boxed.
[b] Nomenclature according to Massung *et al.* (1994), ukn, unknown.

mologs in MCV; F11L is the homolog of MCV018L and O1L is homologous to MCV042L. F5L has no counterpart in MCV, consistent with its nonessential character.

Further significant differences (deletion/insertions > 2 amino acids) in homologous genes located in the central conserved regions of MVA, CPN, and variola are summarized in Table 2. An unusual mutation, the partial deletion of the promoter region of the E8R ORF, may influence expression of this gene in MVA. A second mutation of this type is the deletion of the late part of the hemagglutinin gene promoter (Antoine *et al.*, 1996). Furthermore, some genes have suffered small in-frame deletions, resulting in slightly smaller proteins, among them genes encoding an envelope protein (F3L) and a structural 28-kDa protein (D3R). The F7L gene product, encoding a protein with lys-asn repeats, has suffered an internal deletion of 12 amino acids. A further interesting mutation that may affect the stability of MVA virions is the deletion of 9 amino acids in the membrane-associated core protein A4L (Cudmore *et al.*, 1996).

A point mutation in the first of two possible ATGs of the A24R ORF, encoding the large RNA polymerase subunit, has the consequence that only the major primary gene product of 1155 amino acids, the 132-kDa form of the enzyme (Patel and Pickup, 1989), can be synthesized. A second mutation affecting a start codon was noted; the A22R gene in the CPN strain is mutated, resulting in a protein of reduced size. The MVA and variola homologs share the same initiation codons. Although the functions of many proteins have been elucidated, the majority of genes in the central conserved region have not been characterized in detail.

Deleted and mutated ORFs in the right terminal genomic region

The right terminal region, beginning downstream of the RNA polymerase rpo132 subunit gene (A24R), with the remnants of the CPX A-type inclusion body (ATI) ORF and its flanking regions, is structurally more conserved than the left one. However, three large deletions, termed d3501, d809, and d1771, were found in the right terminal region relative to CPN sequence (Fig. 2). Deletion d3501 has been described previously and includes the ORFs A51R-A55R (Antoine *et al.*, 1996), among them a small ORF (A53R) with homologies to TNFR. Due to d3551, a large new ORF (MVA164R) that is a fusion between ORFs A51R and A55R was formed, resulting in a hybrid gene. The promoter region of the hemagglutinin gene is also affected by d3551, presumably resulting in poor expression of this gene (see also Antoine *et al.*, 1996). Deletion d809 affects the small ORF B20R of unknown function while deletion d1771, located in the ITR, includes the ORFs B25R-B28R, a region fragmented in the CPN strain that corresponds to a large ORF in variola coding for a 69k ankyrin-like gene (BSH-G1R).

An array of more conserved genes, A27L-A38L, is located downstream of the ATI ORFs, including many proteins present in extracellular enveloped virions (EEV). One of them, the 43- to 50-kDa EEV membrane protein encoded by ORF A36R is also a virulence factor and determines plaque size (Parkinson and Smith, 1994). This gene is intact in MVA but two in-frame deletions of 27 and 12 nucleotides result in an altered protein of slightly reduced size, potentially affecting the properties of MVA.

A fragmented ORF that follows this conserved region in MVA is A39R encoding the human semaphorin (SEM) homolog. The semaphorin gene family encodes neural growth cone guidance molecules (see Kolodkin *et al.*, 1993) and was recently also found in lymphoid tissue, including T cells and natural killer cells (Furuyama *et al.*, 1996; Hall *et al.*, 1996). The semaphorin-like genes are structurally intact in CPN but disrupted in the variola strains as well as in MVA. Semaphorins are also present in herpesvirus (Ensser and Fleckenstein, 1995).

The ORF located downstream of the semaphorin homolog, A40R, was originally described as a lectin-like protein (Smith *et al.*, 1991). Recent progress in molecular biology of natural killer (NK) cells (review: Lanier, 1997) allowed the identification of this ORF as the human NK cell receptor (NKCR) homolog; the structures of A40R in various poxviruses and the potential role of this molecule, including MVA immune evasion, is discussed elsewhere (Scheiflinger *et al.*, submitted for publication).

One of the interesting genes downstream of the NK receptor homolog is the profilin homolog A42R (Blasco *et al.*, 1991). This ORF has a 15-nt in-frame deletion in MVA, which may affect the function of the slightly smaller profilin in the microfilament metabolism in which profilins are involved. The adjacent ORF A43R also carries a 12-nt deletion, reducing its size from 194 to 190 amino acids. The 3β-hydroxysteroid dehydrogenase (3β-HSD; A44L) is intact in MVA and CPN, while defective in variola. The superoxide dismutase-like ORF (SOD; A45R) carries an internal 12-nt deletion in MVA relative to the CPN and variola sequences.

A hypervariable region begins downstream of the viral ligase gene (Table 1). The guanylate kinase (GK; A57R) is truncated in MVA while intact in the CPN and variola strains. Further fragmented ORFs in the right terminal genomic region include B2R, an ORF of unknown function, split into two small ORFs (MVA168R and MVA169R), and B4R, a 65kDa ankyrin-like protein, split into the ORFs MVA171R and MVA172R. The B4R protein corresponds to the MT-5 protein, which is a strong virulence factor in myxoma virus (Mossman *et al.*, 1996). Many of the classical poxviral immune evasion genes located in the right terminal region, including the interferon-γ receptor (IFN-γR; B8R), the interleukin converting enzyme inhibitor (SPI-2), the interferon-α/β receptor (IFN-α/βR; B19R) and the TNFR (CPX crmB), are fragmented; the interleukin 1β receptor (IL1β-R), however, is intact and highly conserved between MVA, the WR strain, and CPX (see Table 1).

The right terminal genomic region additionally harbors ankyrin-like genes, the structures of which are summarized in Table 3. With the exception of the 68-kDa ank gene (B18R), all genes of this class are either fragmented or deleted in MVA, among them the 65- and the 54-kDa ank genes B4R and M1L, respectively. Interestingly, a small gene fragment, homologous to the largest poxvirus protein identified so far, the variola transmembrane protein (BSH-B22R), is present in the right terminal region (MVA188R). The last ORF in the unique part of the genome (B22R; unknown function) has suffered two deletions relative to the CPN sequence causing frame shifts. The duplicated open reading frames located in the right ITR are described above.

DISCUSSION

Mutated structural and membrane proteins potentially affecting the physical properties of MVA

Although MVA grows efficiently in chicken embryo fibroblasts (Mayr and Malicki, 1966) and also in baby hamster kidney (BHK) cells (Carroll and Moss, 1997), it seems to be unstable upon purification (F. G. Falkner, unpublished). The reasons for these properties are unclear, but may be the results of mutations in structural and membrane proteins. Most genes encoding structural, membrane, and core proteins are highly conserved among orthopoxviruses. However, several exceptions from this rule were found in the MVA sequence. The A36R ORF (MVA147R) has suffered two internal deletions of 9 and 3 amino acids that may affect EEV formation and virulence (see also Parkinson and Smith, 1994). A structural component that carries an internal deletion of 3 amino acids in MVA is the D3R protein, found in a detergent-insoluble fraction of the virion (Dyster and Niles, 1991). A further interesting structural protein carrying an internal deletion of 9 amino acids is the membrane-associated core protein p39 encoded by ORF A4L. This protein most likely interacts with an integral membrane protein of the IMV and possibly functions as a matrix-like linker protein between the core and the innermost of the two membranes surrounding the IMV (Cudmore *et al.*, 1996). Substitution of the D3R or A4L ORFs by their wild-type counterparts may improve the physical properties of MVA.

Host range genes and genes containing ankyrin repeats in MVA

The "classical" host range genes in orthopoxviruses include K1L and C7L, thought to be sufficient for growth of vaccinia in human cells, and the CPX virus hr gene, which extends the vaccinia host range to Chinese hamster ovary cells (review: Perkus *et al.*, 1990). The structures of the hr and ankyrin-like genes are summarized in Table 3. As described previously (Altenburger *et al.*, 1989; Meyer *et al.*, 1991), the hr gene K1L is partially deleted in

TABLE 3

Structure of ORFs of MVA Located in the Terminal Regions Specifying Potential Host Range (hr) Proteins, Including Proteins with Ankyrin (ank) Repeats and their Homologs in Vaccinia Copenhagen and Variola Virus

MVA[a]	CPN	VAR[b]	Amino acids MVA/CPN/VAR	Putative function/ homology
003L	C17L	—	102/386/—	45k ank[c] protein
004L	C17L	D1L	233/386/91	45k ank protein
009L	—	—	90/—/—	77k CPX[d] hr protein
010L	—	D6L	142/—/452	77k CPX hr protein
011L	—	D6L	135/—/452	77k CPX hr protein
012L	—	D6L	90/—/452	77k CPX hr protein
013L	—	D6L	71/—/452	77k CPX hr protein
014L	C9L	D6.5L	109/634/91	75k ank protein
015L	C9L	—	96/634/—	75k ank protein
016L	C9L	D7L	297/634/153	75k ank protein
018L	C7L	D11L	150/150/150	hr protein
—	M1L	O1L	—/472/446	54k ank protein
022L	K1L	O3L	98/284/70+76	Host range
022L	K1L	C1L	98/284/76	Protein
050L	E3L	E3L	190/190/192	dsRNA dep. PKI
171R	B4R	B5R	177/558/558	65k ank protein
172R	B4R	B5R	409/558/558	65k ank protein
173R	B5R	B6R	317/317/317	ps/hr / EEVgp42
186R	B18R	B16R	574/574/574	68k ank protein
190R	B23R	D1L	233/386/91	45k ank protein
191R	B23R	—	102/386/—	45k ank protein

Note. —, deleted in the respective virus.
[a,b] see Table 2.
[c] ankyrin.
[d] cowpox host range.

MVA, and, similar to the situation in variola, the CPX hr homolog is split into several separate ORFs. Only C7L is intact (100% identity to C7L of the vaccinia WR strain); nevertheless, MVA does not grow in human cells. A further candidate gene that may affect host range in MVA is the serpin gene SPI-1, the lack of which in rabbit poxvirus (a close relative of vaccinia) resulted in a restricted host range including human cells (Ali *et al.*, 1994). The SPI-1 gene is deleted in MVA.

A class of genes related to and including some hr genes are the ankyrin-like (ank) genes (review: Shchelkunov *et al.*, 1993b). All ank genes are defective or deleted in MVA except for the 68-kDa ank gene (B18R), the function of which is unclear. The 54-kDa ank gene (M1L) is deleted in MVA and the 65-kDa ank gene (B4R) is split into two pieces. A homolog of B4R, the strong myxoma virulence factor M-T5 (Mossman *et al.*, 1996), is involved in attenuation and host range in myxoma virus. Two further ank genes present in variola, the 69k and the 94k ank genes (BSH-B18R and BSH-G1R), are absent in MVA. In summary, despite the presence of an intact human host range gene (C7L), MVA does not grow in most mammalian cell lines; replication of vaccinia in human cells is dependent not only on the configuration of the classical human hr genes, but also on the genetic background of the respective vaccinia strain.

A comparison of MVA with the host range restricted vaccinia strain NYVAC, which enharbors 18 engineered deleted ORFs (Tartaglia *et al.*, 1992), revealed that both viruses share common deleted or nonfunctional ORFs including the 6 ORFs of deletion d4817 (C5L-N1L), ORFs B13R and B14R encoding the ICE inhibitor, the ATI remnant ORF A26L, and the K1L host range gene. In contrast to NYVAC, the MVA strain has a functional thymidine kinase gene, an intact C7L host range gene, and intact C6L, A56R (hemagglutinin), and I4L (ribonucleotide reductase) ORFs. Despite similarities, the two potential life vaccine viruses have a clearly different genetic background.

Further ORFs involved in host–virus interaction

Homologies of vaccinia proteins with proteins involved in lipid metabolism were found recently; the vaccinia K4L protein is homologous to the phospholipase D (PLD) gene family (Cao *et al.*, 1997; Sung *et al.*, 1997). Mutation of a crucial motif found in the vaccinia PLD homolog resulted in loss of efficient vaccinia virus cell-to-cell spreading, suggesting that it encodes a lipid modifying or binding activity (Sung *et al.*, 1997). The adjacent ORFs K5L and K6L are fragments of a gene that is intact in cowpox virus and is homologous to human LPL (Table 1).

Lysophosphatidic acid, the product of LPLs, is a multifunctional phospholipid messenger with many biological activities (review: Moolenaar *et al.*, 1997). Lysophospholipases are suspected to be virulence factors in many pathogenic bacteria, such as *Vibrio cholerae* (Whayeb *et al.*, 1996) or *Mycobacterium leprae* (Prabhakaran *et al.*, 1996). The presence of intact PLD homologs (K4L) and the fragmentation of the LPL homologs (K5L and K6L) seem to be markers for attenuated vaccinia viruses. In variola, both proteins are mutated, the K4L homolog is deleted, and the lysophospholipase is fragmented, while in CPX both ORFs are intact.

The role of the semaphorin homologs, further potential host interactive proteins in viruses, is still unclear. Originally identified as molecules expressed in neural tissue (Kolodkin *et al.*, 1993), expression of family members in lymphoid cells was found, suggesting a role in the immune system. It seems reasonable to speculate that an interaction of viral semaphorins with lymphoid, rather than neural, cells occurs. Some semaphorins, such as CD100, interact with the CD40/CD40L B cell signaling system (Hall *et al.*, 1996), which also affects virus replication (Ruby *et al.*, 1995). In T cells and natural killer cells a serine kinase activity is associated with CD100 (Elhabazi *et al.*, 1997). These cell types may be partners for interaction with viral semaphorins. Interestingly, the ORF adjacent to this potential immune modulator is the NK receptor homolog A40R (Table 1). Although key mutations were responsible for the attenuation of MVA (Meyer *et al.*, 1991), numerous genes in MVA that differ only slightly compared to their CPN or BSH counterparts presumably contribute to the properties of MVA. Thus, the highly attenuated phenotype of MVA is the result of numerous mutations including large and small deletions and substitutions, resulting in the deletion and fragmentation of ORFs and the alteration of proteins.

MATERIALS AND METHODS

Sequence analysis of the MVA genome

Prior to sequencing, the MVA strain (obtained from Prof. A. Mayr, University of Munich, Faculty of Veterinary Medicine) was plaque purified once. The DNA from MVA clone M4 was cleaved with *Xba*I and cloned into pUC19 and minipreparations of DNA (QIAprep-8 kit (Qiagen 19047)) were sequenced (Sanger *et al.*, 1977) on an Applied Biosystems Model 373A Sequencer using the cycle sequencing method with dye terminators and AmpliTaq DNA polymerase FS (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit 402122; Perkin–Elmer, Inc.). Selected regions were sequenced with genomic DNA as template with manually designed primers. Frameshift mutations were confirmed by direct sequencing of the corresponding genomic templates. The sequence of the MVA genome was deposited with GenBank (Accession No. U94848) using the Sequin Program (National Center for Biotechnology Information, NIH). Open reading frames (ORFs) >65 codons were translated using the MacMolly sequence analysis Software (Softgene, Inc.) and protein sequences were compared with the nonredundant protein sequence database (National Center for Biotechnology Information, NIH) using the program BLASTp (Altschul *et al.*, 1990). Selected proteins were also compared to the protein databases using the BLAST2 program that constructs alignments with gaps (Altschul and Gish, 1996).

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REFERENCES

Adamkiewicz, T. V., McSherry, C., Bach, F. H., and Houchins, J. P. (1994). Natural killer lectin-like receptors have divergent carboxy-termini, distinct from C-type lectins. *Immunogenetics* 39, 218–218.

Ahn, B. Y., Gershon, P. D., Jones, E. V., and Moss, B. (1990a). Identification of rpo30, a vaccinia virus RNA polymerase gene with structural similarity to a eucaryotic transcription elongation factor. *Mol. Cell. Biol.* 10, 5433–5441.

Ahn, B. Y., Jones, E. V., and Moss, B. (1990b). Identification of the vaccinia virus gene encoding an 18-kilodalton subunit of RNA polymerase and demonstration of a 5′ poly(A) leader on its early transcript. *J. Virol.* 64, 3019–3024.

Ahn, B. Y., and Moss, B. (1992a). Glutaredoxin homolog encoded by vaccinia virus is a virion-associated enzyme with thioltransferase and dehydroascorbate reductase activities. *Proc. Natl. Acad. Sci. USA* 89, 7060–7064.

Ahn, B. Y., and Moss, B. (1992b). RNA polymerase-associated transcription specificity factor encoded by vaccinia virus. *Proc. Natl. Acad. Sci. USA* 89, 3536–3540.

Ahn, B. Y., Rosel, J., Cole, N. B., and Moss, B. (1992). Identification and expression of rpo19, a vaccinia virus gene encoding a 19-kilodalton DNA-dependent RNA polymerase subunit. *J. Virol.* 66, 971–982.

Akopian, T. A., Kaverina, E. N., Naroditsky, B. S., and Tikhonenko, T. I. (1992). Nucleotide sequence analysis of the avian adenovirus CELO (FAV1) DNA fragment (92-100%). *Mol. Gen. Microbiol. Virol.* 11, 19–23.

Alcami, A., and Smith, G. L. (1992). A soluble receptor for interleukin-1 beta encoded by vaccinia virus: A novel mechanism of virus modulation of the host response to infection. *Cell* 71, 153–167.

Alcami, A., and Smith, G. L. (1995). Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. *J. Virol.* 69, 4633–4639.

Ali, A. N., Turner, P. C., Brooks, M. A., and Moyer, R. W. (1994). The SPI-1 gene of rabbitpox virus determines host range and is required for hemorrhagic pock formation. *Virology* 202, 305–314.

Altenburger, W., Suter, C. P., and Altenburger, J. (1989). Partial deletion of the human host range gene in the attenuated vaccinia virus MVA. *Arch. Virol.* 105, 15–27.

Altschul, S. F., and Gish, W. (1996). Local alignment statistics. *Methods Enzymol.* 266, 460–480.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Amegadzie, B. Y., Ahn, B. Y., and Moss, B. (1991a). Identification, sequence, and expression of the gene encoding a Mr 35,000 subunit of the vaccinia virus DNA-dependent RNA polymerase. *J. Biol. Chem.* 266, 13712–13718.

Amegadzie, B. Y., Ahn, B. Y., and Moss, B. (1992). Characterization of a 7-kilodalton subunit of vaccinia virus DNA-dependent RNA polymerase with structural similarities to the smallest subunit of eukaryotic RNA polymerase II. *J. Virol.* 66, 3003–3010.

Amegadzie, B. Y., Holmes, M. H., Cole, N. B., Jones, E. V., Earl, P. L., and Moss, B. (1991b). Identification, sequence, and expression of the gene encoding the second-largest subunit of the vaccinia virus DNA-dependent RNA polymerase. *Virology* 180, 88–95.

Antoine, G., Scheiflinger, F., Holzer, G., Langmann, T., Falkner, F. G., and Dorner, F. (1996). Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes. *Gene* 177, 43–46.

Baker, M. E., and Blasco, R. (1992). Expansion of the mammalian 3 beta-hydroxysteroid dehydrogenase/plant dihydroflavonol reductase superfamily to include a bacterial cholesterol dehydrogenase, a bacterial UDP-galactose-4-epimerase, and open reading frames in vaccinia virus and fish lymphocystis disease virus. *FEBS Lett.* 301, 89–93.

Banham, A. H., and Smith, G. L. (1992). Vaccinia virus gene B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. *Virology* 191, 803–812.

Baylis, C. D., and Smith, G. L. (1997). Vaccinia virion protein VP8, the 25kDa product of the L4R gene, binds single-stranded DNA and RNA with similar affinity. *Nucleic Acids Res.* 25, 3984–3990.

Baylis, S. A., Twigg, S. R., Vydelingum, S., Dixon, L. K., and Smith, G. L. (1993). Three African swine fever virus genes encoding proteins with homology to putative helicases of vaccinia virus. *J. Gen. Virol.* 74, 1969–1974.

Baylis, C. D., and Condit, R. C. (1995). The vaccinia virus A18R gene product is a DNA-dependent ATPase. *J. Biol. Chem.* 270, 1550–1556.

Beattie, E., Tartaglia, J., and Paoletti, E. (1991). Vaccinia virus-encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. *Virology* 183, 419–422.

Bertholet, C., Drillien, R., and Wittek, R. (1985). One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. *Proc. Natl. Acad. Sci. USA* 82, 2096–2100.

Binns, M. M., Boursnell, M. E., Tomley, F. M., and Campbell, J. (1989). Analysis of the fowlpoxvirus gene encoding the 4b core polypeptide and demonstration that it possesses efficient promoter sequences. *Virology* 170, 288–291.

Binns, M. M., Britton, P. S., Mason, C., and Boursnell, M. E. (1990). Analysis of the fowlpox virus genome region corresponding to the vaccinia virus D6 to A1 region: Location of, and variation in, non-essential genes in poxviruses. *J. Gen. Virol.* 71, 2873–2881.

Binns, M. M., Stenzler, L., Tomley, F. M., Campbell, J., and Boursnell, M. E. (1987). Identification by a random sequencing strategy of the fowlpoxvirus DNA polymerase gene, its nucleotide sequence and comparison with other viral DNA polymerases. *Nucleic Acids Res.* 15, 6563–6573.

Binns, M. M., Tomley, F. M., Campbell, J., and Boursnell, M. E. (1988). Comparison of a conserved region in fowlpox virus and vaccinia virus genomes and the translocation of the fowlpox virus thymidine kinase gene. *J. Gen. Virol.* 69, 1275–1283.

Blasco, R., Cole, N. B., and Moss, B. (1991). Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. *J. Virol.* 65, 4598–4608.

Boursnell, M. E., Foulds, I. J., Campbell, J. I., and Binns, M. M. (1988). Non-essential genes in the vaccinia virus HindIII K fragment: A gene related to serine protease inhibitors and a gene related to the 37K vaccinia virus major envelope antigen. *J. Gen. Virol.* 69, 2995–3003.

Brady, W. A., Kokoris, M. S., Fitzgibbon, M., and Black, M. E. (1996). Cloning, characterization, and modeling of mouse and human guanylate kinases. *J. Biol. Chem.* 271, 16734–16740.

Broyles, S. S., and Fesler, B. S. (1990). Vaccinia virus gene encoding a component of the viral early transcription factor. *J. Virol.* 64, 1523–1529.

Broyles, S. S., and Moss, B. (1986). Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: Nucleotide sequence and transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. *Proc. Natl. Acad. Sci. USA* 83, 3141–3145.

Broyles, S. S., and Moss, B. (1987). Identification of the vaccinia virus gene encoding nucleoside triphosphate phosphohydrolase I, a DNA-dependent ATPase. *J. Virol.* 61, 1738–1742.

Broyles, S. S., and Pennington, M. J. (1990). Vaccinia virus gene encoding a 30-kilodalton subunit of the viral DNA-dependent RNA polymerase. *J. Virol.* 64, 5376–5382.

Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., and Venter, J. C. (1996). Complete genome sequence of the methanogenic archeon, *Methanococcus jannaschii*. *Science* 273, 1058–1073.

Buyse, I. M., Shao, G., and Huang, S. (1995). The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A. *Proc. Natl. Acad. Sci. USA* 92, 4467–4467.

Cabirac, G. F., Strayer, D. S., Sell, S., and Leibowitz, J. L. (1985). Characterization, molecular cloning, and physical mapping of the Shope fibroma virus genome. *Virology* 143, 663–670.

Calvert, J. G., Ogawa, R., Yanagida, N., and Nazerian, K. (1992). Identification and functional analysis of the fowlpox virus homolog of the vaccinia virus p37K major envelope antigen gene. *Virology* 191, 783–792.

Campbell, I. G., Freemont, P. S., Foulkes, W., and Trowsdale, J. (1992). An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. *Cancer Res.* 52, 5416–5420.

Cao, J. X., Gershon, P. D., and Black, D. N. (1995). Sequence analysis of HindIII Q2 fragment of capripoxvirus reveals a putative gene encoding a G-protein-coupled chemokine receptor homologue. *Virology* 209, 207–212.

Cao, J. X., Koop, B. F., and Upton, C. (1997). A human homolog of the vaccinia virus HindIII K4L gene is a member of the phospholipase D superfamily. *Virus Res.* 48, 11–18.

Carroll, M. W., and Moss, B. (1997). Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: Propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* 238, 198–205.

Cavallaro, K. F., and Esposito, J. J. (1992). Sequences of the raccoon poxvirus hemagglutinin protein. *Virology* 190, 434–439.

Chang, C., Rodriquez, A., Carretero, M., Lopez-Botet, M., Phillips, J. H., and Lanier, L. L. (1995a). Molecular characterization of human CD94: A type II membrane glycoprotein related to the C-type lectin superfamily. *Eur. J. Immunol.* 25, 2433–2437.

Chang, H. W., Uribe, L. H., and Jacobs, B. L. (1995b). Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. *J. Virol.* 69, 6605–6608.

Chang, H. W., Watson, J. C., and Jacobs, B. L. (1992). The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 89, 4825–4829.

Chang-Yen, A., Mold, D. E., and Huang, R. C. C. (1991). Identification of a novel murine IAP-promoted placenta-expressed gene. *Nucleic Acids Res.* 19, 3667–3672.

Chen, W., Drillien, R., Spehner, D., and Buller, R. M. (1992). Restricted replication of ectromelia virus in cell culture correlates with mutations in virus-encoded host range gene. *Virology* 187, 433–442.

Chertov, O., Telezhinskaya, I. N., Zaitseva, E. V., Golubeva, T. B., Zinov'ev, V. V., Ovechkina, L. G., Mazkova, L. B., and Malygin, E. G. (1991). Amino acid sequence determination of vaccinia virus immunodominant protein p35 and identification of the gene. *Biomed. Sci.* 2, 151–154.

Ciliberto, G., Dente, L., and Cortese, R. (1985). Cell-specific expression of a transfected human alpha1-antitrypsin gene. *Cell*, 531–540.

Colamonici, O. R., Domanski, P., Sweitzer, S. M., Larner, A., and Buller, R. M. (1995). Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. *J. Biol. Chem.* 270, 15974–15978.

Coppola, T., Waldmann, R., Borsotto, M., Heurteaux, C., Romey, G., Mattei, M. G., and Lazdunski, M. (1994). Molecular cloning of a

murine N-type calcium channel alpha1 subunit. Evidence for isoforms, brain distribution, and chromosomal localization. *FEBS Lett.* **338**, 1–5.

Cudmore, S., Blasco, R., Vincentelli, R., Esteban, M., Sodeik, B., Griffiths, G., and Krijnse Locker, J. (1996). A vaccinia virus core protein, p39, is membrane associated. *J. Virol.* **70**, 6909–6921.

Davies, M. V., Elroy-Stein, O., Jagus, R., Moss, B., and Kaufman, R. J. (1992). The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *J. Virol.* **66**, 1943–1950.

Davis, R. E., and Mathews, C. K. (1993). Acidic C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase. *Proc. Natl. Acad. Sci. USA* **90**, 745–749.

Deblandre, G., Marinx, O., Evans, S., Majjaj, S., Leo, O., Caput, D., Huez, G., and Wathelet, M. (1995). Expression cloning of an interferon-inducible 17-kDa membrane protein implicated in the control of cell growth. *J. Biol. Chem.* **270**, 23860–23866.

Demkowicz, W. E., Maa, J. S., and Esteban, M. (1992). Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. *J. Virol.* **66**, 386–398.

Douglas, N. J., and Dumbell, K. R. (1996). DNA sequence variation as a clue to the phylogenesis of orthopoxviruses. *J. Gen. Virol.* **77**, 947–951.

Drillien, R., Spehner, D., Villeval, D., and Lecocq, J. P. (1987). Similar genetic organization between a region of fowlpox virus DNA and the vaccinia virus HindIII J fragment despite divergent location of the thymidine kinase gene. *Virology* **160**, 203–209.

Dubel, S. J., Starr, T. V. B., Hell, J., Ahlijanian, M. K., Enyeart, J. J., Catterall, W. A., and Snutch, T. P. (1992). Molecular cloning of the alpha-1 subunit of an omega-conotoxin-sensitive calcium channel. *Proc. Natl. Acad. Sci. USA* **89**, 5058–5062.

Duncan, S. A., and Smith, G. L. (1992a). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *J. Virol.* **66**, 1610–1621.

Duncan, S. A., and Smith, G. L. (1992b). Vaccinia virus gene SalF5R is non-essential for virus replication in vitro and in vivo. *J. Gen. Virol.* **73**, 1235–1242.

Dyster, L. M., and Niles, E. G. (1991). Genetic and biochemical characterization of vaccinia virus genes D2L and D3R which encode virion structural proteins. *Virology* **182**, 455–467.

Earl, P. L., Jones, E. V., and Moss, B. (1986). Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: Nucleotide sequence of the vaccinia virus DNA polymerase gene. *Proc. Natl. Acad. Sci. USA* **83**, 3659–3663.

Elhabazi, A., Lang, V., Herold, C., Freeman, G. J., Bensussan, A., Boumsell, L., and Bismuth, G. (1997). The human semaphorin-like leukocyte cell surface molecule CD100 associates with a serine kinase activity. *J. Biol. Chem.* **272**, 23515–23520.

Engelstad, M., Howard, S. T., and Smith, G. L. (1992). A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* **188**, 801–810.

Ensser, A., and Fleckenstein, B. (1995). The Alcelaphine herpesvirus type 1 has a semaphorin-like gene. *J. Gen. Virol.* **76**, 1063–1067.

Fernando, M. R., Sumimoto, H., Nanri, H., Kawabata, S., Iwanaga, S., Minakami, S., Fukumaki, Y., and Takeshige, K. (1994). Cloning and sequencing of the cDNA encoding human glutaredoxin. *Biochim. Biophys. Acta* **1218**, 229–231.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., and Merrick, J. M. (1995). Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science* **269**, 496–512.

Fleming, S. B., Blok, J., Fraser, K. M., Mercer, A. A., and Robinson, A. J. (1993). Conservation of gene structure and arrangement between vaccinia virus and orf virus. *Virology* **195**, 175–184.

Franke, C. A., Wilson, E. M., and Hruby, D. E. (1990). Use of a cell-free system to identify the vaccinia virus L1R gene product as the major late myristylated virion protein M25. *J. Virol.* **64**, 5986–5996.

Funahashi, S., Sato, T., and Shida, H. (1988). Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. *J. Gen. Virol.* **69**, 35–47.

Furuyama, T., Inagaki, S., Kosugi, A., Noda, S., Saitoh, S., Ogata, M., Iwahashi, Y., Miyazaki, N., Hamaoka, T., and Tohyama, M. (1996). Identification of a novel transmembrane semaphorin expressed on lymphocytes. *J. Biol. Chem.* **271**, 33376–33381.

Gershon, P. D., Ahn, B. Y., Garfield, M., and Moss, B. (1991). Poly(A) polymerase and a dissociable polyadenylation stimulatory factor encoded by vaccinia virus. *Cell* **66**, 1269–1278.

Gershon, P. D., Ansell, D. M., and Black, D. N. (1989). A comparison of the genome organization of capripoxvirus with that of the orthopoxviruses. *J. Virol.* **63**, 4703–4708.

Gershon, P. D., and Black, D. N. (1989a). A capripoxvirus pseudogene whose only intact homologs are in other poxvirus genomes. *Virology* **172**, 350–354.

Gershon, P. D., and Black, D. N. (1989b). The nucleotide sequence around the capripoxvirus thymidine kinase gene reveals a gene shared specifically with leporipoxvirus. *J. Gen. Virol.* **70**, 525–533.

Gershon, P. D., and Moss, B. (1990). Early transcription factor subunits are encoded by vaccinia virus late genes. *Proc. Natl. Acad. Sci. USA* **87**, 4401–4405.

Gershon, P. D., and Moss, B. (1993). Stimulation of poly(A) tail elongation by the VP39 subunit of the vaccinia virus-encoded poly(A) polymerase. *J. Biol. Chem.* **268**, 2203–2210.

Gillard, S., Spehner, D., Drillien, R., and Kirn, A. (1986). Localization and sequence of a vaccinia virus gene required for multiplication in human cells. *Proc. Natl. Acad. Sci. USA* **83**, 5573–5577.

Giorda, R., Ohmachi, T., and Ennis, H. L. (1989). Organization of a gene family developmentally regulated during *Dictyostelium discoideum* spore germination. *J. Mol. Biol.* **205**, 63–69.

Giorda, R., Weisberg, E. P., Ip, T. K., and Trucco, M. (1992). Genomic structure and strain-specific expression of the natural killer cell receptor NKR-P1. *J. Immunol.* **149**, 1957–1963.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179**, 247–266, 517–563.

Gong, S. C., Lai, C. F., and Esteban, M. (1990). Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. *Virology* **178**, 81–91.

Graham, K. A., Lalani, A. S., Macen, J. L., Ness, T. L., Barry, M., Liu, L. Y., Lucas, A., Clark-Lewis, I., Moyer, R. W., and McFadden, G. (1997). The T1/35kDa family of poxvirus-secreted proteins bind chemokines and modulate leukocyte influx into virus-infected tissues. *Virology* **229**, 12–24.

Grosenbach, D. W., Ulaeto, D. O., and Hruby, D. E. (1997). Palmitylation of the vaccinia virus 37-kDa major envelope antigen. Identification of a conserved acceptor motif and biological relevance. *J. Biol. Chem.* **272**, 1956–1964.

Guan, K. L., Broyles, S. S., and Dixon, J. E. (1991). A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature* **350**, 359–362.

Gum, J. R. J., Hicks, J. W., Toribara, N. W., Kim, Y. S., and Siddiki, B. (1994). Molecular cloning of human intestinal mucin (MUC2) cDNA: Identification of the amino terminal and overall sequence similarity to pre-pro-von Willebrand factor. *J. Biol. Chem.* **269**, 2440–2446.

Gvakharia, B. O., Koonin, E., and Mathews, C. (1996). Vaccinia virus G4L gene encodes a second glutaredoxin. *Virology* **226**, 408–411.

Hall, K. T., Boumsell, L., Schultze, J. L., Boussiotis, V. A., Dorfman, D. M., Cardoso, A. A., Bensussan, A., Nadler, L. M., and Freeman, G. J. (1996). Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. *Proc. Natl. Acad. Sci. USA* **93**, 11780–11785.

Hall, R. L., and Moyer, R. W. (1991). Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia virus-related open reading frames. *J. Virol.* **65**, 6516–6527.

Hamann, J., Fiebig, H., and Strauss, M. (1993). Molecular cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain. *J. Immunol.* 150, 4920–4927.

Hansen, M., Albers, M., Backes, U., Coblenz, A., Leutner, H., Neu, R., Schreer, A., Schafer, B., Zimmerman, M., and Wolf, K. (1996). The sequence of a 23.4 kb segment on the right arm of chromosome VII from *Saccharomyces cerevisiae* reveals CLB6, SPT6, RP28A and NUP57 genes, a Ty3 element and 11 new open reading frames. *Yeast* 12, 1273–1277.

Heller, R. A., Song, K., Onasch, M. A., Fischer, W. H., Chang, D., and Ringold, G. M. (1990). Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor. *Proc. Natl. Acad. Sci. USA* 87, 6151–6155.

Hirt, P., Hiller, G., and Wittek, R. (1986). Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. *J. Virol.* 58, 757–764.

Hooda-Dhingra, U., Patel, D. D., Pickup, D. J., and Condit, R. C. (1990). Fine structure mapping and phenotypic analysis of five temperature-sensitive mutations in the second largest subunit of vaccinia virus DNA-dependent RNA polymerase. *Virology* 174, 60–69.

Juchins, J. P., Yabe, T., McSherry, C., and Bach, F. H. (1991). DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J. Exp. Med.* 173, 1017–1020.

Howard, S. T., Chan, Y. S., and Smith, G. L. (1991). Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* 180, 633–647.

Howard, S. T., and Smith, G. L. (1989). Two early vaccinia virus genes encode polypeptides related to protein kinases. *J. Gen. Virol.* 70, 3187–3201.

Hruby, D. E., and Ball, L. A. (1982). Mapping and identification of the vaccinia virus thymidine kinase gene. *J. Virol.* 43, 403–409.

Hu, F. Q., and Pickup, D. J. (1991). Transcription of the terminal loop region of vaccinia virus DNA is initiated from the telomere sequences directing DNA resolution. *Virology* 181, 716–720.

Hu, F. Q., Smith, C. A., and Pickup, D. J. (1994). Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* 204, 343–356.

Huang, M. E., Chuat, J. C., and Galibert, F. (1994). A possible yeast homolog of human active-gene-repairing helicase ERCC6. *Biochem. Biophys. Res. Commun.* 201, 310–317.

Ison, R. A., Zhou, Y., Collins, M. D., Johnson, E. A., Hatheway, C. L., and Sugiyama, H. (1996). Genetic characterization of *Chlostridium botulinum* type A containing silent type B neurotoxin gene sequences. *J. Biol. Chem.* 271, 10786–10792.

Isaacs, S. N., Wolffe, E. J., Payne, L. G., and Moss, B. (1992). Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *J. Virol.* 66, 7217–7224.

Jackson, R. J., and Bults, H. G. (1990). A myxoma virus nucleotide sequence with homology to the vaccinia virus RNA polymerase 22-kDa subunit gene. *Nucleic Acids Res.* 18, 5290.

Jackson, R. J., and Bults, H. G. (1992). The myxoma virus thymidine kinase gene: sequence and transcriptional mapping. *J. Gen. Virol.* 73, 323–328.

Jackson, R. J., Hall, D. F., and Kerr, P. J. (1996). Construction of recombinant myxoma viruses expressing foreign genes from different intergenic sites without associated attenuation. *J. Gen. Virol.* 77, 1569–1575.

Jensen, O. N., Houthaeve, T., Shevchenko, A., Cudmore, S., Ashford, T., Mann, M., Griffiths, G., and Krijnse Locker, J. (1996). Identification of the major membrane and core proteins of vaccinia virus by two-dimensional electrophoresis. *J. Virol.* 70, 7485–7497.

Johnson, G. P., Goebel, S. J., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1991). Vaccinia virus encodes a protein with similarity to glutaredoxins. *Virology* 181, 378–381.

Kane, E. M., and Shuman, S. (1992). Temperature-sensitive mutations in the vaccinia virus H4 gene encoding a component of the virion RNA polymerase. *J. Virol.* 66, 5752–5762.

Kane, E. M., and Shuman, S. (1993). Vaccinia virus morphogenesis is blocked by a temperature-sensitive mutation in the I7 gene that encodes a virion component. *J. Virol.* 67, 2689–2698.

Kao, S. Y., and Bauer, W. R. (1987). Biosynthesis and phosphorylation of vaccinia virus structural protein VP11. *Virology* 159, 399–407.

Katsanis, N., Fitzgibbon, J., and Fisher, E. M. C. (1996). Paralogy mapping: Identification of a region in the human MHC triplicated onto human chromosomes 1 and 9 allows the prediction and isolation of novel PBX and NOTCH loci. *Genomics* 35, 101–108.

Keck, J. G., Baldick, C. J., Jr., and Moss, B. (1990). Role of DNA replication in vaccinia virus gene expression: A naked template is required for transcription of three late trans-activator genes. *Cell* 61, 801–809.

Keck, J. G., Kovacs, G. R., and Moss, B. (1993). Overexpression, purification, and late transcription factor activity of the 17-kilodalton protein encoded by the vaccinia virus A1L gene. *J. Virol.* 67, 5740–5748.

Kerr, S. M., and Smith, G. L. (1989). Vaccinia virus encodes a polypeptide with DNA ligase activity. *Nucleic Acids Res.* 17, 9039–9050.

Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. (1994). Molecular cloning of cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc. Natl. Acad. Sci. USA* 91, 11457–11461.

Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* 75, 1389–1399.

Koonin, E. V. (1993). A highly conserved sequence motif defining the family of MutT-related proteins from eubacteria, eukaryotes and viruses. *Nucleic Acids Res.* 21, 4847.

Koonin, E. V., and Senkevich, T. G. (1992). Vaccinia virus encodes four putative DNA and/or RNA helicases distantly related to each other. *J. Gen. Virol.* 73, 989–993.

Koonin, E. V., Senkevich, T. G., and Chernos, V. I. (1993). Gene A32 product of vaccinia virus may be an ATPase involved in viral DNA packaging as indicated by sequence comparisons with other putative viral ATPases. *Virus Genes* 7, 89–94.

Kotwal, G. J. (1996). The great escape. Immune evasion by pathogens. *Immunologist* 4/5, 157–164.

Kotwal, G. J., and Moss, B. (1988a). Analysis of a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. *Virology* 167, 524–537.

Kotwal, G. J., and Moss, B. (1988b). Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* 335, 176–178.

Kotwal, G. J., and Moss, B. (1989). Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. *J. Virol.* 63, 600–606. [Published erratum appears in *J. Virol.*, 1990, 64(2), 966]

Kovacs, G. R., and Moss, B. (1996). The vaccinia virus H5R gene encodes late gene transcription factor 4: purification, cloning, and overexpression. *J. Virol.* 70, 6796–6802.

Krijnse-Locker, J., Schleich, S., Rodriguez, D., Goud, B., Snijder, E. J., and Griffiths, G. (1996). The role of a 21-kDa viral membrane protein in the assembly of vaccinia virus from the intermediate compartment. *J. Biol. Chem.* 271, 14950–14958.

Kumar, S., and Boyle, D. B. (1990). Mapping of a major early/late gene of fowlpox virus. *Virus Res.* 15, 175–186.

Kwiatkowski, D. J., and Bruns, G. A. P. (1988). Human profilin: Molecular cloning sequence comparison, and chromosomal analysis. *J. Biol. Chem.* 263, 5910–5915.

Ladner, R. D., McNulty, D. E., Carr, S. A., and Roberts, G. D. (1996). Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. *J. Biol. Chem.* 271, 7745–7751.

Lanier, L. L. (1997). Natural killer cell receptors and MHC class I interactions. *Curr. Opin. Immunol.* 9, 126–131.

Lin, S., and Broyles, S. S. (1994). Vaccinia protein kinase 2: A second essential serine/threonine protein kinase encoded by vaccinia virus. *Proc. Natl. Acad. Sci. USA* 91, 7653–7657.

Lin, S., Chen, W., and Broyles, S. S. (1992). The vaccinia virus B1R gene product is a serine/threonine protein kinase. *J. Virol.* 66, 2717–2723.

Lindberg, F. P., Gresham, H. D., Schwarz, E., and Brown, E. J. (1993). Molecular cloning of integrin-associated protein: An immunoglobulin family member with multiple membrane spanning domains implicated in alpha-v beta-3-dependent ligand binding. *J. Cell. Biol.* 123, 485–496.

Loskutoff, D. J., Linders, M., Keuer, J., Veerman, H., van Heerikhuizen, H., and Pannekoek, H. (1987). Structure of the human plasminogen activator inhibitor 1 gene. Nonrandom distribution of introns. *Biochemistry* 26, 3763–3768.

Maa, J. S., Rodriguez, J. F., and Esteban, M. (1990). Structural and functional characterization of a cell surface binding protein of vaccinia virus. *J. Biol. Chem.* 265, 1569–1577.

Martin, K. H., Grosenbach, D. W., Franke, C. A., and Hruby, D. E. (1997). Identification and analysis of three myristylated vaccinia virus late proteins. *J. Virol.* 71, 5216–5226.

Massung, R. F., Jayarama, V., and Moyer, R. W. (1993). DNA sequence analysis of conserved and unique regions of swinepox virus: Identification of genetic elements supporting phenotypic observations including a novel G protein-coupled receptor homologue. *Virology* 197, 511–528.

Massung, R. F., Liu, L. I., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C., and Esposito, J. J. (1994). Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975. *Virology* 201, 215–240.

Massung, R. F., Loparev, V. N., Knight, J. C., Totmenin, A. V., Chizhikov, V. E., Parsons, J. M., Safronov, P. F., Gutorov, V. V., Shchelkunov, S. N., and Esposito, J. J. (1996). Terminal region sequence variations in variola virus DNA. *Virology* 221, 291–300.

Massung, R. F., McFadden, G., and Moyer, R. W. (1992). Nucleotide sequence analysis of a unique near-terminal region of the tumorigenic poxvirus, Shope fibroma virus. *J. Gen. Virol.* 73, 2903–2911.

Mayr, A., and Malicki, K. (1966). Attenuierung von virulentem Hühnerpockenvirus in Zellkulturen und Eigenschaften des attenuierten Virus. *Zentralb. Vet. Med. B.* 13, 1–13.

Mayr, A., Stickl, H., Müller, H. K., Danner, K., and Singer, H. (1978). The smallpox vaccination strain MVA: Marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism. *Zbl. Bakt. Hyg. I.Abt. Orig. B* 167, 375–390.

McIntosh, A. A., and Smith, G. L. (1996). Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus. *J. Virol.* 70, 272–281.

McMahan, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., and Brannan, C. I. (1991). A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J.* 10, 2821–2832.

Meis, R. J., and Condit, R. C. (1991). Genetic and molecular biological characterization of a vaccinia virus gene which renders the virus dependent on isatin-beta-thiosemicarbazone (IBT). *Virology* 182, 442–454.

Mercer, A. A., Fraser, K. M., Stockwell, P. A., and Robinson, A. J. (1989). A homologue of retroviral pseudoproteases in the parapoxvirus, orf virus. *Virology* 172, 665–668.

Mercer, A. A., Green, G., Sullivan, J. T., Robinson, A. J., and Drillien, R. (1996). Location, DNA sequence and transcriptional analysis of the DNA polymerase gene of orf virus. *J. Gen. Virol.* 77, 1563–1568.

Mercer, A. A., Lyttle, D. J., Whelan, E. M., Fleming, S. B., and Sullivan, J. T. (1995). The establishment of a genetic map of orf virus reveals a pattern of genomic organization that is highly conserved among divergent poxviruses. *Virology* 212, 698–704.

Merchlinsky, M., and Moss, B. (1989). Nucleotide sequence required for resolution of the concatemer junction of vaccinia virus DNA. *J. Virol* 63, 4354–4361.

Messmer, B., and Dreyer, C. (1993). Requirements for nuclear translocation and nucleolar accumulation of nucleolin of *X. laevis*. *Eur. J. Cell Biol.* 61, 369–382.

Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* 62, 379–390.

Meyer, H., Osterrieder, N., and Czerny, C. P. (1994). Identification of binding sites for neutralizing monoclonal antibodies on the 14-kDa fusion protein of orthopox viruses. *Virology* 200, 778–783.

Meyer, H., and Rziha, H. J. (1993). Characterization of the gene encoding the A-type inclusion protein of camelpox virus and sequence comparison with other orthopoxviruses. *J. Gen. Virol.* 74, 1679–1684.

Meyer, H., Sutter, G., and Mayr, A. (1991). Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 72, 1031–1038.

Miller, C. G., Shchelkunov, S. N., and Kotwal, G. J. (1997). The cowpox virus-encoded homolog of the vaccinia virus complement control protein is an inflammation modulatory protein. *Virology* 229, 126–133.

Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. M. (1997). Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.* 9, 168–173.

Moore, J. B., and Smith, G. L. (1992). Steroid hormone synthesis by a vaccinia enzyme: A new type of virus virulence factor *EMBO J.* 11, 1973–1980. [Published erratum appears in *EMBO J.* 1992, 11(9), 3490]

Morgan, J. R., Cohen, L. K., and Roberts, B. E. (1984). Identification of the DNA sequences encoding the large subunit of the mRNA-capping enzyme of vaccinia virus. *J. Virol.* 52, 206–214.

Moss, B. (1996). Poxviridae: The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, J. Melnick, B. Roizman, and R. Shope, Eds.). Raven Press, Philadelphia.

Mossman, K., Lee, S. F., Barry, M., Boshkov, L., and McFadden, G. (1996). Disruption of M-T5, a novel myxoma virus gene member of the poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. *J. Virol.* 70, 4394–4411.

Mossman, K., Ostergaard, H., Upton, C., and McFadden, G. (1995a). Myxoma virus and Shope fibroma virus encode dual-specificity tyrosine/serine phosphatases which are essential for virus viability. *Virology* 206, 572–582.

Mossman, K., Upton, C., Buller, R. M., and McFadden, G. (1995b). Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. *Virology* 208, 762–769.

Mustafa, A., and Yuen, L. (1991). Identification and sequencing of the *Choristoneura biennis* entomopoxvirus DNA polymerase gene. *DNA Seq.* 2, 39–45.

Naase, M., Nicholson, B. H., Fraser, K. M., Mercer, A. A., and Robinson, A. J. (1991). An orf virus sequence showing homology to the 14K 'fusion' protein of vaccinia virus. *J. Gen. Virol.* 72, 1177–1181.

Neumann, H., and Zillig, W. (1990). Nucleotide sequence of the viral protein TPX of the TTV1 variant VT3. *Nucleic Acids Res.* 18, 2171.

Niles, E. G., Condit, R. C., Caro, P., Davidson, K., Matusick, L., and Seto, J. (1986). Nucleotide sequence and genetic map of the 16-kb vaccinia virus *Hin*dIII D fragment. *Virology* 153, 96–112.

Niles, E. G., Lee-Chen, G. J., Shuman, S., Moss, B., and Broyles, S. S. (1989). Vaccinia virus gene D12L encodes the small subunit of the viral mRNA capping enzyme. *Virology* 172, 513–522.

Niles, E. G., and Seto, J. (1988). Vaccinia virus gene D8 encodes a virion transmembrane protein. *J. Virol.* 62, 3772–3776.

Nishiyama, Y., Tanaka, T., Naitoh, H., Mori, C., Fukumoto, M., Hiai, H., and Toyokuni, S. (1997). Overexpression of integrin-associated protein (CD47) in rat kidney treated with a renal carcinogen, ferric nitrilotriacetate. *Jpn. J. Cancer Res.* 88, 120–128.

O'Connell, M. A., Krause, S., Higuchi, M., Hsuan, J. J., Totty, N. F., Jenny, A., and Keller, W. (1995). Cloning of cDNAs encoding mammalian double-stranded RNA-specific adenosine deaminase. *Mol. Cell. Biol.* 15, 1389–1397.

Ogawa, R., Calvert, J. G., Yanagida, N., and Nazerian, K. (1993). Insertional inactivation of a fowlpox virus homologue of the vaccinia virus

F12L gene inhibits the release of enveloped virions. *J. Gen. Virol.* 74, 55–64.

Chaba..., Bailey, L. C., Attree, O., Srinivasan, S., Perkel, J. M., Laurent, B. C., Carlson, M., Nelson, D. L., and Nussbaum, R. L. (1992). Cloning of human and bovine homologs of SNF2/SWI2: A global activator of transcription in yeast *S. cerevisiae*. *Nucleic Acids Res.* 20, 4649–4655.

Osborne, R. J., Symonds, T. M., Sriskantha, A., Lai-Fook, J., Fernon, C. A., and Dall, D. J. (1995). An entomopoxvirus homologue of the vaccinia virus D13L-encoded rifampicin resistance protein. *J. Gen. Virol.* 77, 839–846.

Parkinson, J. E., and Smith, G. L. (1994). Vaccinia virus gene A36R encodes a M(r) 43-50 K protein on the surface of extracellular enveloped virus. *Virology* 204, 376–390.

Parks, R. J., Lichty, B. D., Karakis, C., and Evans, D. H. (1994). Characterization of the Shope fibroma virus DNA ligase gene. *Virology* 202, 642–650.

Passarelli, A. L., Kovacs, G. R., and Moss, B. (1996). Transcription of a vaccinia virus late promoter template: Requirement for the product of the A2L intermediate-stage gene. *J. Virol.* 70, 4444–4450.

Patel, A. H., Gaffney, D. F., Subak-Sharpe, J. H., and Stow, N. D. (1990). DNA sequence of the gene encoding a major secreted protein of vaccinia virus, strain Lister. *J. Gen. Virol.* 71, 2013–2021.

Patel, D. D., and Pickup, D. J. (1989). The second-largest subunit of the poxvirus RNA polymerase is similar to the corresponding subunits of procaryotic and eucaryotic RNA polymerases. *J. Virol.* 63, 1076–1086.

Pena, L., Yanez, R. J., Revilla, Y., Vinuela, E., and Salas, M. L. (1993). African swine fever virus guanylyltransferase. *Virology* 193, 319–328.

Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E. (1990). Vaccinia virus host range genes. *Virology* 179, 276–286.

Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Norton, E. K., and Paoletti, E. (1991). Deletion of 55 open reading frames from the termini of vaccinia virus. *Virology* 180, 406–410.

Peters, L. L., John, K. M., Lu, F. M., Eicher, E. M., Higgins, A., Yialamas, M., Turtzo, L. C., Otsuka, A. J., and Lux, S. E. (1995). Ank3 (epithelial ankyrin), a widely distributed new member of the ankyrin gene family and the major ankyrin in kidney, is expressed in alternatively spliced forms, including forms that lack the repeat domain. *J. Cell Biol.* 130, 313–330.

Pickup, D. J., Ink, B. S., Hu, W., Ray, C. A., and Joklik, W. K. (1986). Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. *Proc. Natl. Acad. Sci. USA* 83, 7698–7702.

Pluciennicczak, A., Schroeder, E., Zettlmeissl, G., and Streeck, R. E. (1985). Nucleotide sequence of a cluster of early and late genes in a conserved segment of the vaccinia virus genome. *Nucleic Acids Res.* 13, 985–998.

Prabhakaran, K., Harris, E. B., and Randhawa, B. (1996). Properties of lysophospholipase in *Mycobacterium leprae*. *J. Basic Microbiol.* 36, 341–349.

Print, C., Leung, E., Harrison, J., and Watoson, J. (1994). Cloning of a gene encoding a human leukocyte protein characterized by extensive heptad repeats. *Gene* 144, 221–228.

Quick, S. D., and Broyles, S. S. (1990). Vaccinia virus gene D7R encodes a 20,000-dalton subunit of the viral DNA-dependent RNA polymerase. *Virology* 178, 603–605.

Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvensen, G. S., and Pickup, D. J. (1992). Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell* 69, 597–604.

Reppert, S. M., Weaver, D. R., Cassone, V. M., Godson, C., and Kolakowski, L. F. (1995). Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron* 15, 1003–1015.

Rodriguez, D., Esteban, M., and Rodriguez, J. R. (1995). Vaccinia virus A17L gene product is essential for an early step in virion morphogenesis. *J. Virol.* 69, 4640–4648.

Rodriguez, J. F., and Esteban, M. (1987). Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. *J. Virol.* 61, 3550–3554.

Rodriguez, J. F., Kann, J. S., and Esteban, M. (1956). Molecular cloning, encoding sequence, and expression of vaccinia virus nucleic acid-dependent nucleoside triphosphatase gene. *Proc. Natl. Acad. Sci. USA* 83, 9566–9570.

Rodriguez, J. F., and Smith, G. L. (1990). IPTG-dependent vaccinia virus: Identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res.* 18, 5347–5351.

Roper, R. L., Payne, L. G., and Moss, B. (1996). Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. *J. Virol.* 70, 3753–3762.

Rosel, J., and Moss, B. (1985). Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b. *J. Virol.* 56, 830–838.

Rosel, J. L., Earl, P. L., Weir, J. P., and Moss, B. (1986). Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment. *J. Virol.* 60, 436–449.

Roseman, N. A., Evans, R. K., Mayer, E. L., Rossi, M. A., and Slabaugh, M. B. (1996). Purification and characterization of the vaccinia virus deoxyuridine triphosphatase expressed in *Escherichia coli*. *J. Biol. Chem.* 271, 23506–23511.

Roseman, N. A., and Slabaugh, M. B. (1990). The vaccinia virus *HindIII* F fragment: Nucleotide sequence of the left 6.2 kb. *Virology* 178, 410–418.

Ruby, J., Bluethmann, H., Aguet, M., and Ramshaw, I. A. (1995). CD40 ligand has potent antiviral activity. *Nature Med.* 1, 437–441. [See comments]

Safronov, P. F., Petrov, N. A., Riazankina, O. I., Totmenin, A. V., Shchelkunov, S. N., and Sandakhiev, L. S. (1996). Genes of a circle of hosts for the cowpoxvirus. *Dokl. Akad. Nauk.* 349, 829–833.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.

Schmitt, J. F., and Stunnenberg, H. G. (1988). Sequence and transcriptional analysis of the vaccinia virus HindIII I fragment. *J. Virol.* 62, 1889–1897.

Schmutz, C., Payne, L. G., Gubser, J., and Wittek, R. (1991). A mutation in the gene encoding the vaccinia virus 37,000-M(r) protein confers resistance to an inhibitor of virus envelopment and release. *J. Virol.* 65, 3435–3442.

Schneider, S. S., Schick, C., Fish, K. E., Miller, E., Pena, J. C., Treter, S., Hui, S. M., and Silverman, G. A. (1995). A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene. *Proc. Natl. Acad. Sci. USA* 92, 3147–3151.

Schnitzlein, W. M., and Tripathy, D. N. (1991). Identification and nucleotide sequence of the thymidine kinase gene of swinepox virus. *Virology* 181, 727–732.

Schweizer, M., and Neumann-Haefelin, D. (1995). Phylogenetic analysis of primate foamy virus by comparison of pol sequences. *Virology* 207, 577–582.

Senkevich, T. G., Bugert, J. J., Sisler, J. R., Koonin, E. V., Darai, G., and Moss, B. (1996). Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. *Science* 273, 813–816.

Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. (1997). The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. *Virology* 233, 19–42.

Senkevich, T. G., Koonin, E. V., and Buller, R. M. (1993a). A poxvirus protein with a RING zinc finger motif is of crucial importance for virulence. *Virology* 198, 118–128.

Senkevich, T. G., Muravnik, G. L., Pozdnyakov, S. G., Chizhkov, V. E., Ryazankina, O. I., Shchelkunov, S. N., Koonin E. V., and Chernos, V. I.

(1993b). Nucleotide sequence of XhoI O fragment of ectromelia virus DNA reveals significant differences from vaccinia virus. *Virus Res.* 30, 73–88.

Seralini, G. E., Underhill, C. M., Smith, C. L., Nguyen, V. T., and Hammond, G. L. (1989). Biological half-life and transfer of maternal corticosteroid-binding globulin to amniotic fluid in the rabbit. *Endocrinology* 125, 1321–1325.

Shchelkunov, S. N., Blinov, V. M., Resenchuk, S. M., Totmenin, A. V., and Sandakhchiev, L. S. (1993a). Analysis of the nucleotide sequence of a 43 kbp segment of the genome of variola virus India-1967 strain. *Virus Res.* 30, 239–258.

Shchelkunov, S. N., Blinov, V. M., and Sandakhchiev, L. S. (1993b). Ankyrin-like proteins of variola and vaccinia viruses. *FEBS Lett.* 319, 163–165.

Shchelkunov, S. N., Blinov, V. M., Totmenin, A. V., Marennikova, S. S., Kolykhalov, A. A., Frolov, I. V., Chiznikov, V. E., Gytorov, V. V., Gashikov, P. V., and Belanov, E. F. (1993c). Nucleotide sequence analysis of variola virus HindIII M, L, I genome fragments. *Virus Res.* 27, 25–35.

Shchelkunov, S. N., Marennikova, S. S., Blinov, V. M., Resenchuk, S. M., Totmenin, A. V., Chizhikov, V. E., Guturov, V. V., Safronov, P. F., Kurmanov, R. K., and Sandakhchiev, L. S. (1993d). Entire coding sequence of the variola virus. *Dokl. Akad. Nauk.* 328, 629–632.

Shchelkunov, S. N., Massung, R. F., and Esposito, J. J. (1995). Comparison of the genome DNA sequences of Bangladesh-1975 and India-1967 variola viruses. *Virus Res.* 36, 107–118.

Shida, H. (1986). Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* 150, 451–462.

Shuman, S. (1992). Vaccinia virus RNA helicase: An essential enzyme related to the DE-H family of RNA-dependent NTPases. *Proc. Natl. Acad. Sci. USA* 89, 10935–10939.

Shuman, S., and Moss, B. (1987). Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. *Proc. Natl. Acad. Sci. USA* 84, 7478–7482.

Skinner, M. A., Moore, J. B., Binns, M. M., Smith, G. L., and Boursnell, M. E. (1994). Deletion of fowlpox virus homologues of vaccinia virus genes between the 3 beta-hydroxysteroid dehydrogenase (A44L) and DNA ligase (A50R) genes. *J. Gen. Virol.* 75, 2495–2498.

Slabaugh, M., Roseman, N., Davis, R., and Mathews, C. (1988). Vaccinia virus-encoded ribonucleotide reductase: Sequence conservation of the gene for the small subunit and its amplification in hydroxyurea-resistant mutants. *J. Virol.* 62, 519–527.

Smith, G. L., and Chan, Y. S. (1991). Two vaccinia virus proteins structurally related to the interleukin-1 receptor and the immunoglobulin superfamily. *J. Gen. Virol.* 72, 511–518.

Smith, G. L., Chan, Y. S., and Howard, S. T. (1991). Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. *J. Gen. Virol.* 72, 1349–1376.

Smith, G. L., Howard, S. T., and Chan, Y. S. (1989). Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. *J. Gen. Virol.* 70, 2333–2343.

Spehner, D., Gillard, S., Drillien, R., and Kirn, A. (1988). A cowpox virus gene required for multiplication in Chinese hamster ovary cells. *J. Virol.* 62, 1297–1304.

Spriggs, M. K., Hruby, D. E., Maliszewski, C. R., Pickup, D. J., Sims, J. E., Buller, R. M., and VanSlyke, J. (1992). Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein. *Cell* 71, 145–152.

Stamenkovic, I., Clark, E. A., and Seed, B. (1989). A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J.* 8, 1403–1410.

Strayer, D. S., Jerng, H. H., and O'Connor, K. (1991). Sequence and analysis of a portion of the genomes of Shope fibroma virus and malignant rabbit fibroma virus that is important for viral replication in lymphocytes. *Virology* 185, 585–595.

Stroobant, P., Rice, A. P., Gullick, W. J., Cheng, D. J., Kerr, I. M., and Waterfield, M. D. (1985). Purification and characterization of vaccinia virus growth factor. *Cell* 42, 383–393.

Sullivan, J. T., Fleming, S. B., Robinson, A. J., Mercer, A. A. (1995a). Sequence and transcriptional analysis of a near-terminal region of the orf virus genome. *Virus Genes* 11, 21–29.

Sullivan, J. T., Fraser, K. M., Fleming, S. B., Robinson, A. J., and Mercer, A. A. (1995b). Sequence and transcriptional analysis of an orf virus gene encoding ankyrin- like repeat sequences. *Virus Genes* 9, 277–282.

Sullivan, J. T., Mercer, A. A., Fleming, S. B., and Robinson, A. J. (1994). Identification and characterization of an orf virus homologue of the vaccinia virus gene encoding the major envelope antigen p37K. *Virology* 202, 968–973.

Sung, T. C., Roper, R. L., Zhang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J., and Frohman, M. A. (1997). Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. *EMBO J.* 16, 4519–4530.

Sutter, G., and Moss, B. (1992). Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* 89, 10847–10851.

Sutter, G., Wyatt, L. S., Foley, P. L., Bennink, J. R., and Moss, B. (1994). A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 12, 1032–1040.

Symons, J. A., Alcami, A., and Smith, G. L. (1995). Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell* 81, 551–560.

Takahashi, F., Oie, M., and Ichihashi, Y. (1994). N-terminal amino acid sequences of vaccinia virus structural proteins. *Virology* 202, 844–852.

Takahashi-Nishimaki, F., Funahashi, S., Miki, K., Hashizume, S., and Sugimoto, M. (1991). Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. *Virology* 181, 158–164.

Talbott, R. L., Sparger, E. E., Lovelace, K. M., Fitch, W. M., Pedersen, N. C., Luciw, P. A., and Elder, J. H. (1989). Nucleotide sequence and genomic organization of feline immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 86, 5743–5747.

Tamin, A., Esposito, J., and Hruby, D. (1991). A single nucleotide substitution in the 5'-untranslated region of the vaccinia N2L gene is responsible for both alpha-amanitin-resistant and temperature-sensitive phenotypes. *Virology* 182, 393–396.

Tan, J. L., and Spudich, J. A. (1990). Developmentally regulated protein-tyrosine kinase genes in *Dictyostelium discoideum*. *Mol. Cell. Biol.* 10, 3578–3583.

Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J. C., Cox, W. I., Davis, S. W., van der Hoeven, J., Meignier, B., Riviere, M., Languet, B., and Paoletti, E. (1992). NYVAC: A highly attenuated strain of vaccinia virus. *Virology* 188, 217–232.

Tartaglia, J., and Paoletti, E. (1985). Physical mapping and DNA sequence analysis of the rifampicin resistance locus in vaccinia virus. *Virology* 147, 394–404.

Tartaglia, J., Winslow, J., Goebel, S., Johnson, G. P., Taylor, J., and Paoletti, E. (1990). Nucleotide sequence analysis of a 10.5 kbp HindIII fragment of fowlpox virus: Relatedness to the central portion of the vaccinia virus HindIII D region. *J. Gen. Virol.* 71, 1517–1524.

Tengelsen, L. A., Slabaugh, M. B., Bibler, J. K., and Hruby, D. E. (1988). Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus. *Virology* 164, 121–131.

Tomley, F., Binns, M., Campbell, J., and Boursnell, M. (1988). Sequence analysis of an 11.2 kilobase, near-terminal, BamHI fragment of fowlpox virus. *J. Gen. Virol.* 69, 1025–1040.

Twardzik, D. R., Brown, J. P., Ranchalis, J. E., Todaro, G. J., and Moss, B. (1985). Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. *Proc. Natl. Acad. Sci. USA* 82, 5300–5304.

Ueda, Y., Morikawa, S., and Matsuura, Y. (1990). Identification and nucleotide sequence of the gene encoding a surface antigen, induced by vaccinia virus. *Virology* 177, 586–594.

Upton, C., DeLange, A. M., and McFadden, G. (1987). Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. *Virology* **160**, 29–39.

Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991a). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor family that contributes to viral virulence. *Virology* **184**, 370–382.

Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990a). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**, 618–631.

Upton, C., Mossman, K., and McFadden, G. (1992). Encoding of a homolog of the IFN-g receptor by myxoma virus. *Science* **258**, 1369–1372.

Upton, C., Opgenorth, A., Traktman, P., and McFadden, G. (1990b). Identification and DNA sequence of the Shope fibroma virus DNA topoisomerase gene. *Virolcgy* **176**, 439–447.

Upton, C., Stuart, D., and McFadden, G. (1991b). Identification and DNA sequence of the large subunit of the capping enzyme from Shope fibroma virus. *Virology* **183**, 773–777.

Upton, C., Stuart, D. T., and McFadden, G. (1993). Identification of a poxvirus gene encoding a uracil DNA glycosylase. *Proc. Natl. Acad. Sci. USA* **90**, 4518–4522.

Van Meir, E., and Wittek, R. (1988). Fine structure of the vaccinia virus gene encoding the precursor of the major core protein 4a. *Arch. Virol.* **102**, 19–27.

Vanslyke, J. K., Whitehead, S. S., Wilson, E. M., and Hruby, D. E. (1991). The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: A degenerate conserved cleavage motif within core proteins. *Virology* **183**, 467–478.

Vassalli, J. D., Huarte, J., Bosco, D., Sappino, A. P., Sappino, N., Velardi, A., Wohlwend, A., Erno, H., Monard, D., and Belin, D. (1993). Protease-nexin 1 as an androgen-dependent secretory product of the murine seminal vesicle. *EMBO J.* **12**, 1871–1898.

Venkatesan, S., Gershowitz, A., and Moss, B. (1982). Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the inverted terminal repetition. *J. Virol.* **44**, 637–646.

Vos, J. C., Sasker, M., and Stunnenberg, H. G. (1991). Vaccinia virus capping enzyme is a transcription initiation factor. *EMBO J.* **10**, 2553–2558.

Vydelingum, S., Baylis, S. A., Bristow, C., Smith, G. L., and Dixon, L. K. (1993). Duplicated genes within the variable right end of the genome of a pathogenic isolate of African swine fever virus. *J. Gen. Virol.* **74**, 2125–2130.

Wang, S., and Shuman, S. (1995). Vaccinia virus morphogenesis is blocked by temperature-sensitive mutations in the F10 gene, which encodes protein kinase 2. *J. Virol.* **69**, 6376–6388.

Nay, M., Sanders, M., Chatel, M., Tu, Y. H., Knight, A., and Matsudaira, P. (1995). Beta-scruin, a homolog of the actin crosslinking protein scruin, is localized to the acrosomal vesicle of Limulus sperm. *J. Cell Sci.* **108**, 3155–3162.

Wei, Y. F., Robins, P., Carter, K., Caldecott, K., Pappin, D. J. C., Yu, G. L., Wang, R. P., Shell, B. K., Nash, R., Schar, P., Barnes, D. E., Haseltine, W. A., and Lindahl, T. Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination. *Mol. Cell. Biol.* **15**, 3206–3216.

Weinrich, S. L., and Hruby, D. E. (1986). A tandemly-oriented late gene cluster within the vaccinia virus genome. *Nucleic Acids Res.* **14**, 3003–3016.

Weir, J. P., and Moss, B. (1983). Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutation. *J. Virol.* **46**, 530–537.

Whayeb, S. H., Yamamoto, K., Tojo, H., and Honda, T. (1996). Genetic analysis of the chromosomal region encoding lysophospholipase L2 of Vibrio cholerae O1. *Biochim. Biophys. Acta* **1300**, 1–4.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershaw, J., Kirsten, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Shownkeen, R., Smaldon, N., Smith, A., Sonnhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Waterston, R., Watson, A., Weinstock, L., Wilkinson-Sproat, J., and Wohldman, P. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32–38.

Wolffe, E. J., Katz, E., Weisberg, A., and Moss, B. (1997). The A34R glycoprotein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. *J. Virol.* **71**, 3904–3915.

Wolffe, E. J., Moore, D. M., Peters, P. J., and Moss, B. (1996). Vaccinia virus A17L open reading frame encodes an essential component of nascent viral membranes that is required to initiate morphogenesis. *J. Virol.* **70**, 2797–2808.

Wright, C. F., Keck, J. G., Tsai, M. M., and Moss, B. (1991). A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. *J. Virol.* **65**, 3715–3720.

Xue, F., and Cooley, L. (1993). Kelch encodes a component of intercellular bridges in Drosophila egg chambers. *Cell* **72**, 681–693.

Yanez, R. J., Rodriquez, J. M., Nogal, M. L., Yuste, L., Enriques, C., Rodriguez, J. F., and Vinuela, E. (1993). Analysis of the complete nucleotide sequence of African Swine Fever Virus. *Virology* **208**, 249–278.

Yang, W. P., and Bauer, W. R. (1988). Purification and characterization of vaccinia virus structural protein VP8. *Virology* **167**, 578–584.

Yuen, L., Noiseux, M., and Gomes, M. (1991). DNA sequence of the nucleoside triphosphate phosphohydrolase I (NPH I) of the *Chordoneura biennis* entomopoxvirus. *Virology* **182**, 403–406.

Zantinge, J. L., Krell, P. J., Derbyshire, J. B., and Nagy, E. (1996). Partial transcriptional mapping of the fowlpox virus genome and analysis of the EcoRI L fragment. *J. Gen. Virol.* **77**, 603–614.

Zhang, H., Scheirer, D. C., Fowle, W. H., and Goodman, H. M. (1992). Expression of antisense gene blocks chloroplast differentiation in Arabidopsis. *Plant Cell* **4**, 1575–1588.